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(57) Abstract

The invention relates to recombinant polypeptides and peptides and particularly to the polypeptide containing in its polypeptidic chain the following amino acid sequence: the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (194) represented in fig. 4a and fig. 4b. The polypeptides and peptides of the invention can be used for the diagnostic of tuberculosis, and can also be part of the active principle in the preparation of vaccine against tuberculosis.

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RECOMBINANT POLYPEPTIDES AND PEPTIDES, NUCLEIC ACIDS CODING FOR THE SAME AND USE OF THESE POLYPEPTIDES AND PEPTIDES IN THE DIAGNOSTIC OF TUBERCULOSIS

The invention relates to recombinant polypeptides and peptides, which can be used for the diagnosis of tuberculosis. The invention also relates to a process for preparing the above-said polypeptides and peptides, which are in a state of biological purity such that they can be used as part of the active principle in the preparation of vaccines against tuberculosis.

It also relates to nucleic acids coding for said polypeptides and peptides.

Furthermore, the invention relates to the <u>in vitro</u> diagnostic methods and kits using the above-said polypeptides and peptides and to the vaccines containing the above-said polypeptides and peptides as active principle against tuberculosis.

By "recombinant polypeptides or peptides" it is to be understood that it relates to any molecule having a polypeptidic chain liable to be produced by genetic engineering, through transcription and translation, of a corresponding DNA sequence under the control of appropriate regulation elements within an efficient cellular host. Consequently, the expression "recombinant polypeptides" such as is used herein does not exclude the possibility for the polypeptides to comprise other groups, such as glycosylated groups.

The term "recombinant" indeed involves the fact that the polypeptide has been produced by genetic engineering, particularly because it results from the expression in a cellular host of the corresponding nucleic acid sequences which have previously been introduced into the expression vector used in said host.

Nevertheless, it must b understood that this expression does not exclud th possibility for the polypeptide to be produced by a different process, for instance by classical chemical synthesis according to methods used in the protein synthesis or by proteolytic cleavage of larger molecules.

The expression "biologically pure" or "biological purity" means on the one hand a grade of purity such that the recombinant polypeptide can be used for the production of vaccinating compositions and on the other hand the absence of contaminants, more particularly of natural contaminants.

Tuberculosis remains a major disease in developing countries. The situation is dramatic in some countries, particularly where high incidence of tuberculosis among AIDS patients represents a new source of dissemination of the disease.

Tuberculosis is a chronic infectious disease in which cell-mediated immune mechanisms play an essential role both for protection against and control of the disease.

Despite BCG vaccination, and some effective drugs, tuberculosis remains a major global problem. Skin testing with tuberculin PPD (protein-purified derivative) largely used for screening of the disease is poorly specific, due to cross reactivity with other pathogenic or environmental saprophytic mycobacteria.

Moreover, tuberculin PPD when used in serological tests (ELISA) does not allow to discriminate between patients who have been vaccinated by BCG, or those who have been primo-infected, from those who are developing evolutive tuberculosis and for whom an early and rapid diagnosis would be necessary.

A protein with a molecular weight of 32-kDa has been purified (9) from zinc deficient Mycobacterium bovis BCG culture filtrat (8). This 32-kDa protein of

M. bovis BCG has been purified from Sauton zinc deficient cultur filtrat of M. bovis BCG using successively hydrophobic chromatography on Phenyl-Sepharose, ion exchange on DEAE-Sephacel and molecular sieving on Sephadex G-100. The final preparation has been found to be homogeneous as based on several analyses. This P_{32} protein is a constituent of BCG cells grown in normal conditions. It represents about 3% of the soluble fraction of a cellular extract, and appears as the major protein released in normal Sauton culture filtrate. This protein has been found to have a molecular weight of 32 000 by SDS-polyacrylamide gel electrophoresis and by molecular sieving.

The NH_2-terminal amino acid sequence of the 32-kDa protein of M. bovis BCG (Phe-Ser-Arg-Pro-Gly-Leu) is identical to that reported for the MPB 59 protein purified from M. bovis BCG substrain Tokyo (34).

Purified P_{32} of M. bovis BCG has been tested by various cross immunoelectrophoresis techniques, and has been shown to belong to the antigen 85 complex in the reference system for BCG antigens. It has been more precisely identified as antigen 85A in the closs reference system for BCG antigens (7).

Increased levels of immunoglobulin G antibodies towards the 32-kDa protein of M. bovis BCG could be detected in 70% of tuberculous patients (30).

Furthermore, the 32-kDa protein of M. bovis BCG induces specific lymphoproliferation and interferon-(IFN-γ) production in peripheral blood leucocytes from patients with active tuberculosis (12) and PPD-positive healthy subjects. Recent findings indicate that the amount of 32-kDa protein of M. bovis BCG-induced IFN-γ in BCG-sensitized mouse spleen cells is under probable H-2 control (13). Finally, the high affinity of mycobact ria for fibronectin is related to proteins of the BCG 85 antigen complex (1).

Matsuo et al. (17) recently cloned the gene encoding the antigen α, a major protein secreted by BCG (substrain Tokyo) and highly homologous to MPB 59 antigen in its NH_2-terminal amino acid sequence, and even identical for its first 6 amino acids : Phe-Ser-Arg-Pro-Gly-Leu.

This gene was cloned by using a nucleotide probe homologous to the N-terminal amino acid sequence of antigen α, purified from M. tuberculosis as described in Tasaka, H. et al., 1983. "Purification and antigenic specificity of alpha protein (Yoneda and Fukui) from Mycobacterium tuberculosis and Mycobacterium intracellulare. Hiroshima J. Med. Sci. 32, 1-8.

The presence of antigens of around 30-32-kDa, named antigen 85 complex, has been revealed from electrophoretic patterns of proteins originating from culture media of mycobacteria, such as Mycobacterium tuberculosis. By immunoblotting techniques, it has been shown that these antigens cross-react with rabbit sera raised against the 32-kDa protein of BCG (8).

A recent study reported on the preferential humoral response to a 30-kDa and 31-kDa antigen in lepromatous leprosy patients, and to a 32-kDa antigen in tuberculoid leprosy patients (24).

It has also been found that fibronectin (FN)-binding antigens are prominent components of short-term culture supernatants of Mycobacterium tuberculosis. In 3-day-old supernatants, a 30-kilodalton (kDa) protein was identified as the major (FN)-binding molecule. In 21-day-old supernatants, FN was bound to a double protein band of around 30 to 32-kDa, as well as to a group of antigens of larger molecular mass (57 to 60 kDa)(1).

In other experiments, recombinant plasmids containing DNA from Mycobacterium tuberculosis were transformed into Escherichia coli, and three colonies

were selected by their reactivity with polyclonal antisera to M. tub rculosis. Each recombinant produced 35- and 53-kilodalton proteins (35K and 53K prot ins, respectively)("Expression of Proteins of Mycobacterium tuberculosis in Escherichia coli and Potential of Recombinant Genes and Proteins for Development of Diagnostic Reagents", Mitchell L Cohen et al., Journal of Clinical Microbiology, July 1987, p.1176-1180).

Concerning the various results known to date, the physico-chemical characteristics of the antigen P_{32} of Mycobacterium tuberculosis are not precise and, furthermore, insufficient to enable its unambiguous identifiability, as well as the characterization of its structural and functional elements.

Moreover, the pathogenicity and the potentially infectious property of M. tuberculosis has hampered research enabling to identify, purify and characterize the constituents as well as the secretion products of this bacteria.

An aspect of the invention is to provide recombinant polypeptides which can be used as purified antigens for the detection and control of tuberculosis.

Another aspect of the invention is to provide nucleic acids coding for the peptidic chains of biologically pure recombinant polypeptides which enable their preparation on a large scale.

Another aspect of the invention is to provide antigens which can be used in serological tests as an in vitro rapid diagnostic of tuberculosis.

Another aspect of the invention is to provide a rapid in vitro diagnostic means for tuberculosis, enabling it to discriminate between patients suffering from an evolutive tuberculosis from those who have been vaccinated against BCG or who have been primo-infected.

Anoth r aspect of the invention is to provide nucleic prob s which can be used as in vitro diagnostic

reagent for tuberculosis, as well as in vitro diagnostic reagent for identifying M. tuberculosis from other strains of mycobacteria.

The recombinant polypeptides of the invention contain in their polypeptidic chain one at least of the following amino acid sequences:

- the one extending from the extremity constituted by amino acid at position (-29) to the extremity constituted by amino acid at position (-1) represented on fig. 3a and fig. 3b, or
- the one extending from the extremity constituted by amino acid at position (12) to the extremity constituted by amino acid at position (31) represented on fig. 3a and fig. 3b, or
- the one extending from the extremity constituted by amino acid at position (36) to the extremity constituted by amino acid at position (55) represented on fig. 3a and fig. 3b, or
- the one extending from the extremity constituted by amino acid at position (77) to the extremity constituted by amino acid at position (96) represented on fig. 3a and fig. 3b, or
- the one extending from the extremity constituted by amino acid at position (101) to the extremity constituted by amino acid at position (120) represented on fig. 3a and fig. 3b, or
- the one extending from the extremity constituted by amino acid at position (175) to the extremity constituted by amino acid at position (194) represented on fig. 3a and fig. 3b, or
- the one extending from the extremity constituted by amino acid at position (211) to the extremity constituted by amino acid at position (230) represented on fig. 3a and fig. 3b, or
- the one extending from the extremity constituted by amino acid at position (275) to th extremity

constituted by amino acid at position (294) represented on fig. 3a and fig. 3b,

and the peptidic sequences resulting from the modification by substitution and/or by addition and/or by deletion of one or several amino acids in so far as this modification does not alter the following properties :

the polypeptides react with rabbit polyclonal antiserum raised against the protein of 32-kDa of M. bovis BCG culture filtrate, and/or

react selectively with human sera from tuberculosis patients and particularly patients developing an evolutive tuberculosis at an early stage,

and/or react with the amino acid sequence extending from the extremity constituted by amino acid at position (1), to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b.

On figures 3a and 3b :

- X represents G or GG,
- Y represents C or CC,
- Z represents C or G,
- W represents C or G and is different from Z,
- K represents C or CG,
- L represents G or CC,
- a_1-b_1 represents ALA-ARG or GLY-ALA-ALA,
- a_2 represents arg or gly,
- a_3-b_3-c_3-d_3-e_3-f_3- represents his-trp-val-pro-arg-pro or ala-leu-gly-ala,
- a_4 represents pro or pro-asn-thr,
- a_5 represents pro or ala-pro.

The recombinant polypeptides of the invention contain in their polypeptidic chain one at least of the following amin acid sequences:

- the one extending from the extremity constituted by amino acid at position (-29) to the extremity constituted by amino acid at position (-1) repr sented on fig. 4a and fig. 4b, or
- the one extending from the extremity constituted by amino acid at position (12) to the extremity constituted by amino acid at position (31) represented on fig. 4a and fig. 4b, or
- the one extending from the extremity constituted by amino acid at position (36) to the extremity constituted by amino acid at position (55) represented on fig. 4a and fig. 4b, or
- the one extending from the extremity constituted by amino acid at position (77) to the extremity constituted by amino acid at position (96) represented on fig. 4a and fig. 4b, or
- the one extending from the extremity constituted by amino acid at position (101) to the extremity constituted by amino acid at position (120) represented on fig. 4a and fig. 4b, or
- the one extending from the extremity constituted by amino acid at position (175) to the extremity constituted by amino acid at position (194) represented on fig. 4a and fig. 4b, or
- the one extending from the extremity constituted by amino acid at position (211) to the extremity constituted by amino acid at position (230) represented on fig. 4a and fig. 4b, or
- the one extending from the extremity constituted by amino acid at position (275) to the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b,

and the peptidic sequences resulting from the modification by substitution and/or by addition and/or by deletion of one or several amino acids in so far as

this modification does not alter the following properties :

the polypeptides react with rabbit polyclonal antis rum raised against the protein of 32-kDa of M. bovis BCG culture filtrate, and/or

react selectively with human sera from tuberculosis patients and particularly patients developing an evolutive tuberculosis at an early stage,

and/or react with the amino acid sequence extending from the extremity constituted by amino acid at position (1), to the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b.

The recombinant polypeptides of the invention contain in their polypeptidic chain one at least of the following amino acid sequences:

- the one extending from the extremity constituted by amino acid at position (-30) to the extremity constituted by amino acid at position (-1) represented on fig. 5, or
- the one extending from the extremity constituted by amino acid at position (12) to the extremity constituted by amino acid at position (31) represented on fig. 5, or
- the one extending from the extremity constituted by amino acid at position (36) to the extremity constituted by amino acid at position (55) represented on fig. 5, or
- the one extending from the extremity constituted by amino acid at position (77) to the extremity constituted by amino acid at position (96) represented on fig. 5, or
- the one extending from the extremity constituted by amino acid at position (101) to the extremity c nstituted by amino acid at position (120) represented on fig. 5, or

- the one extending from the extremity constituted by amino acid at position (175) to th extremity constituted by amino acid at positi n (194) represented on fig. 5, or
- the one extending from the extremity constituted by amino acid at position (211) to the extremity constituted by amino acid at position (230) represented on fig. 5, or
- the one extending from the extremity constituted by amino acid at position (275) to the extremity constituted by amino acid at position (295) represented on fig. 5,

and the peptidic sequences resulting from the modification by substitution and/or by addition and/or by deletion of one or several amino acids in so far as this modification does not alter the following properties :

the polypeptides react with rabbit polyclonal antiserum raised against the protein of 32-kDa of M. bovis BCG culture filtrate, and/or

react selectively with human sera from tuberculosis patients and particularly patients developing an evolutive tuberculosis at an early stage,

and/or react with the amino acid sequence extending from the extremity constituted by amino acid at position (1), to the extremity constituted by amino acid at position (295) represented on fig. 5.

Advantageous polypeptides of the invention are characterized by the fact that they react with rabbit polyclonal antiserum raised against the protein of 32-kDa of M. bovis BCG culture filtrate, hereafter designated by "P_{32} protein of BCG".

Advantageous polypeptides of the invention are characterized by the fact that they selectively react with human sera from tuberculous patients and

particularly patients developing an evolutive tubercul sis at an early stage.

Hereafter is given, in a non limitative way a process for preparing rabbit polyclonal antiserum raised against the P_{32} protein of BCG and a test for giving evidence of the reaction between the polypeptides of the invention and said rabbit polyclonal antiserum raised against the P_{32} protein of BCG.

1) process for preparing rabbit polyclonal antiserum raised against the P_{32} protein of BCG:

Purified P_{32} protein of BCG from culture filtrate is used.

a) Purification of protein P_{32} of BCG :

P_{32} protein can be purified as follows :

The bacterial strains used are M. bovis BCG substrains 1173P2 (Pasteur Institute, Paris) and GL2 (Pasteur Institute, Brussels).

The culture of bacteria is obtained as follows :

. Mycobacterium bovis BCG is grown as a pellicle on Sauton medium, at 37.5°C for 14 days. As the medium is prepared with distilled water, zinc sulfate is added to the final concentration of 5 µM (normal Sauton medium)(De Bruyn J., Weckx M., Beumer-Jochmans M.-P. Effect of zinc deficiency on Mycobacterium tuberculosis var. bovis (BCG). J. Gen. Microbiol. 1981; 124:353-7). When zinc deficient medium was needed, zinc sulfate is omitted.

The filtrates from zinc deficient cultures are obtained as follows :

The culture medium is clarified by decantation. The remaining bacteria are removed by filtration through Millipak 100 filter unit (Millipore Corp., Bedford, Mass.). When used for purification, the filtrat is adjust d to 20 mM in phosphate, 450 mM in NaCl, 1 mM in EDTA, and the pH is brought to 7.3 with

5 M HCl before sterile filtration.

The protein analysis is carried out by polyacrylamide gel electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was done on 13% (w/v) acrylamide-containing gels as described by Laemmli UK. (Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227:680-5). The gels are stained with Coomassie Brilliant Blue R-250 and for quantitative analysis, scanned at 595 nm with a DU8 Beckman spectrophotometer. For control of purity the gel is revealed with silver stain (Biorad Laboratories, Richmond, Calif.).

The purification step of P_{32} is carried out as follows:

Except for hydrophobic chromatography on Phenyl-Sepharose, all buffers contain Tween 80 (0.005% final concentration). The pH is adjusted to 7.3 before sterilization. All purification steps are carried out at +4°C. Elutions are followed by recording the absorbance at 280 nm. The fractions containing proteins are analysed by SDS-PAGE.

(i) The treated filtrate from a 4 liters zinc-deficient culture, usually containing 125 to 150 mg protein per liter, is applied to a column (5.0 by 5.0 cm) of Phenyl-Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden), which is previously equilibrated with 20 mM phosphate buffer (PB) containing 0.45 M NaCl and 1 mM EDTA, at a flow rate of 800 ml per hour. The gel is then washed with one column volume of the same buffer to remove unfixed material and successively with 300 ml of 20 mM and 4 mM PB and 10% ethanol (v/v). The P_{32} appears in the fraction eluted with 10% ethanol.

(ii) After the phosphate concentration of this fraction has been brought to 4 mM, it is applied to a column (2.6 by 10 cm) of DEAE-Sephacel (Pharmacia Fine

Chemicals), which is equilibrated with 4 mM PB. After washing with the equilibrating buffer the sampl is eluted with 25 mM phosphate at a flow rate of 50 ml per hour. The eluate is concentrated in a 202 Amicon stirred cell equipped with a PM 10 membrane (Amicon Corp., Lexington, Mass.).

(iii) The concentrated material is submitted to 4 mg of P_{32} protein of BCG (soluble extract) or molecular sieving on a Sephadex G-100 (Pharmacia) column (2.6 by 45 cm) equilibrated with 50 mM PB, at a flow rate of 12 ml per hour. The fractions of the peak giving one band in SDS-PAGE are pooled. The purity of the final preparation obtained is controlled by SDS-PAGE followed by silverstaining and by molecular sieving on a Superose 12 (Pharmacia) column (12.0 by 30 cm) equilibrated with 50 mM PB containing 0.005% Tween 80 at a flow rate of 0.2 ml/min. in the Fast Protein Liquid Chromatography system (Pharmacia). Elution is followed by recording the absorbance at 280 nm and 214 nm.

b) Preparation of rabbit polyclonal antiserum raised against the P_{32} protein of BCG :

400 µg of purified P_{32} protein of BCG per ml physiological saline are mixed with one volume of incomplete Freund's adjuvant. The material is homogenized and injected intradermally in 50 µl doses delivered at 10 sites in the back of the rabbits, at 0, 4, 7 and 8 weeks (adjuvant is replaced by the diluent for the last injection). One week later, the rabbits are bled and the sera tested for antibody level before being distributed in aliquots and stored at -80°C;

2) test for giving evidence of the reaction between the polypeptides of the invention and said rabbit polyclonal antiserum raised against the P_{32} protein of BCG:

the test used was an ELISA test; the ELISA for antibody d termination is based on the method of Engvall and Perlmann (Engvall, E., and P. Perlmann. 1971. Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry 8:871-874)

Immulon Microelisa plates (Dynatech, Kloten, Switzerland) are coated by adding to each well 1 μg of one of the polypeptides of the invention in 100 μl Tris hydrochloride buffer 50 mM (pH 8.2). After incubation for 2 h at 27°C in a moist chamber, the plates are kept overnight at 4°C. They are washed four times with 0.01 M phosphate-buffered saline (pH 7.2) containing 0.05% Tween 20 by using a Titertek microplate washer (Flow Laboratories. Brussels. Belgium). Blocking is done with 0.5% gelatin in 0.06 M carbonate buffer (pH 9.6) for 1 h. Wells are then washed as before, and 100 μl of above mentioned serum diluted in phosphate-buffered saline containing 0.05% Tween 20 and 0.5% gelatin is added. According to the results obtained in preliminary experiments, the working dilutions are set at 1:200 for IgG, 1:20 for IgA and 1:80 for IgM determinations. Each dilution is run in duplicate. After 2 h of incubation and after the wells are washed, they are filled with 100 μl of peroxidase-conjugated rabbit immunoglobulins directed against human IgG, IgA or IgM (Dakopatts, Copenhagen, Denmark), diluted 1:400, 1:400 and 1:1.200, respectively in phosphate-buffered saline containing 0.05% Tween 20 and 0.5% gelatin and incubated for 90 min. After the wash, the amount of peroxidase bound to the wells is quantified by using a freshly prepared solution of o-phenylenediamine (10 mg/100 ml) and hydrogen peroxide (8μl of 30% H_2O_2 per 100 ml) in 0.15 M citrate buffer (pH 5.0) as a substrate. The enzymatic reaction is stopped with 8 N H_2SO_4 after

15 min. of incubation. Th optical density is r ad at 492 nm with a Tit rtek Multiskan photomet r (Flow Laboratories).

Wells without sera are used as controls for the conjugates. Each experiment is done by including on each plate one negative and two positive reference sera with medium and low antibody levels to correct for plate-to-plate and day-to-day variations. The antibody concentrations are expressed as the optical density values obtained after correction of the readings according to the mean variations of the reference sera.

Hereafter is also given in a non limitative way, a test for giving evidence of the fact that polypeptides of the invention are recognized selectively by human sera from tuberculous patients.

This test is an immunoblotting (Western blotting) analysis, in the case where the polypeptides of the invention are obtained by recombinant techniques. This test can also be used for polypeptides of the invention obtained by a different preparation process. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, polypeptides of the invention are blotted onto nitrocellulose membranes (Hybond C. (Amersham)) as described by Towbin et al. (29). The expression of polypeptides of the invention fused to β-galactosidase in E. coli Y1089, is visualized by the binding of a polyclonal rabbit anti-32-kDa BCG protein serum (1:1,000) or by using a monoclonal anti-β-galactosidase antibody (Promega). The secondary antibody (alkaline phosphatase anti-rabbit immunoglobulin G and anti-mouse alkaline phosphatase immunoglobulin G conjugates, respectively) is diluted as recommended by the supplier (Promega).

In order to identify selective recognition of polypeptid s of the invention and of fusion proteins of the invention by human tuberculous sera, nitrocellulose

sheets are incubat d ov rnight with thes s ra (1:50) (after blocking aspecific protein-binding sites). The human tuberculous sera are selected for their reactivity (high or low) against the purified 32-kDa antigen of BCG tested in a dot blot assay as described in document (31) of the bibliography hereafter. Reactive areas on the nitrocellulose sheets are revealed by incubation with peroxidase conjugated goat anti-human immunoglobulin G antibody (Dakopatts, Copenhagen, Denmark)(1:200) for 4h, and after repeated washings, color reaction is developed by adding peroxidase substrate (α-chloronaphtol)(Bio-Rad Laboratories, Richmond, Calif.) in the presence of peroxidase and hydrogen peroxide.

It goes without saying that the free reactive functions which are present in some of the amino acids, which are part of the constitution of the polypeptides of the invention, particularly the free carboxyl groups which are carried by the groups Glu or by the C-terminal amino acid on the one hand and/or the free NH_2 groups carried by the N-terminal amino acid or by amino acid inside the peptidic chain, for instance Lys, on the other hand, can be modified in so far as this modification does not alter the above mentioned properties of the polypeptide.

The molecules which are thus modified are naturally part of the invention. The above mentioned carboxyl groups can be acylated or esterified.

Other modifications are also part of the invention. Particularly, the amine or ester functions or both of terminal amino acids can be themselves involved in the bond with other amino acids. For instance, the N-terminal amino acid can be linked to a sequence comprising from 1 to several amino acids corresponding to a part of the C-terminal region of another peptid .

Furthermore, any peptidic sequences resulting from the modification by substitution and/or by addition and/or by deletion of one or several amino acids of th polypeptides according to the invention are part of the invention in so far as this modification does not alter the above mentioned properties of said polypeptides.

The polypeptides according to the invention can be glycosylated or not, particularly in some of their glycosylation sites of the type Asn-X-Ser or Asn-X-Thr, X representing any amino acid.

Advantageous recombinant polypeptides of the invention contain in their polypeptidic chain, one at least of the following amino acid sequences:

- the one extending from the extremity constituted by amino acid at position (-42) to the extremity constituted by amino acid at position (-1) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-47) to the extremity constituted by amino acid at position (-1) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-49) to to the extremity constituted by amino acid at position (-1) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-55) to the extremity constituted by amino acid at position (-1) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-59) to the extremity constituted by amino acid at position (-1) represented on fig. 3a and fig. 3b.

Advantageous recombinant polypeptides of the invention contain in their polypeptidic chain, on at least of the f llowing amino acid sequences:

- the one extending from the extremity constituted by amino acid at position (-42) to th extremity constituted by amino acid at position (-1) represented on fig. 4a and fig. 4b,
- the one extending from the extremity constituted by amino acid at position (-47) to the extremity constituted by amino acid at position (-1) represented on fig. 4a and fig. 4b,
- the one extending from the extremity constituted by amino acid at position (-49) to to the extremity constituted by amino acid at position (-1) represented on fig. 4a and fig. 4b,
- the one extending from the extremity constituted by amino acid at position (-55) to the extremity constituted by amino acid at position (-1) represented on fig. 4a and fig. 4b,
- the one extending from the extremity constituted by amino acid at position (-59) to the extremity constituted by amino acid at position (-1) represented on fig. 4a and fig. 4b.

Advantageous recombinant polypeptides of the invention contain in their polypeptidic chain, one at least of the following amino acid sequences:
- the one extending from the extremity constituted by amino acid at position (-43) to the extremity constituted by amino acid at position (-1) represented on fig. 5.

Advantageous recombinant polypeptides of the invention contain in their polypeptidic chain, one at least of the following amino acid sequences:
- the one extending from the extremity constituted by amino acid at position (1) to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amin acid at position (-29) to th extremity

constituted by amino acid at position (294) represented on fig. 3a and fig. 3b,

- the one extending from the extremity constituted by amino acid at position (-42) to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-47) to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-49) to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-55) to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-59) to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b.

Advantageous recombinant polypeptides of the invention contain in their polypeptidic chain, one at least of the following amino acid sequences:

- the one extending from the extremity constituted by amino acid at position (1) to the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b,
- the one extending from the extremity constituted by amino acid at position (-29) to the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b,
- the one extending from the extremity constituted by amin acid at positi n (-42) to th extremity

constituted by amino acid at position (294) represented on fig. 4a and fig. 4b,

- the one extending from the extremity constituted by amino acid at position (-47) to the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b,
- the one extending from the extremity constituted by amino acid at position (-49) to the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b,
- the one extending from the extremity constituted by amino acid at position (-55) to the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b,
- the one extending from the extremity constituted by amino acid at position (-59) to the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b.

Advantageous recombinant polypeptides of the invention contain in their polypeptidic chain, one at least of the following amino acid sequences:

- the one extending from the extremity constituted by amino acid at position (1) to the extremity constituted by amino acid at position (295) represented on fig. 5,
- the one extending from the extremity constituted by amino acid at position (-30) to the extremity constituted by amino acid at position (295) represented on fig. 5,
- the one extending from the extremity constituted by amino acid at position (-43) to the extremity constituted by amino acid at position (295) represented on fig. 5.

Other advantageous recombinant polypeptides of the invention consist in one of the following amino acid sequences:

- th one extending from the extremity constituted by amino acid at position (-59) to the extremity constituted by amino acid at position (294) r presented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-55) to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-49) to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-47) to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-42) to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-29) to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (1) to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b.

Other advantageous recombinant polypeptides of the invention consist in one of the following amino acid sequences:

- the one extending from the extremity constituted by amino acid at position (-59) to the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b,

- the one xtending from the extremity constitut d by amino acid at position (-55) t the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b,
- the one extending from the extremity constituted by amino acid at position (-49) to the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b,
- the one extending from the extremity constituted by amino acid at position (-47) to the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b,
- the one extending from the extremity constituted by amino acid at position (-42) to the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b,
- the one extending from the extremity constituted by amino acid at position (-29) to the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b,
- the one extending from the extremity constituted by amino acid at position (1) to the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b.

Other advantageous recombinant polypeptides of the invention consist in one of the following amino acid sequences:

- the one extending from the extremity constituted by amino acid at position (1) to the extremity constituted by amino acid at position (295) represented on fig. 5,
- the one extending from the extremity constituted by amino acid at position (-30) to the extremity constituted by amino acid at position (295) represented on fig. 5,
- the one extending from the extremity constituted by amin acid at position (-43) to the extremity

constituted by amino acid at position (295) repr sented on fig. 5.

Other advantageous recombinant polypeptides of the invention consist in one of the following amino acid sequences:

- the one extending from the extremity constituted by amino acid at position (-59) to the extremity constituted by amino acid at position (-1) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-55) to the extremity constituted by amino acid at position (-1) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-49) to the extremity constituted by amino acid at position (-1) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-47) to the extremity constituted by amino acid at position (-1) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-42) to the extremity constituted by amino acid at position (-1) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-29) to the extremity constituted by amino acid at position (-1) represented on fig. 3a and fig. 3b.

Other advantageous recombinant polypeptides of the invention consist in one of the following amino acid sequences:

- the one extending from the extremity constituted by amino acid at position (-59) to the extremity constituted by amino acid at position (-1) represented on fig. 4a and fig. 4b,

- the one extending from the extremity constituted by amin acid at position (-55) to the extremity constituted by amino acid at position (-1) represented on fig. 4a and fig. 4b,
- the one extending from the extremity constituted by amino acid at position (-49) to the extremity constituted by amino acid at position (-1) represented on fig. 4a and fig. 4b,
- the one extending from the extremity constituted by amino acid at position (-47) to the extremity constituted by amino acid at position (-1) represented on fig. 4a and fig. 4b,
- the one extending from the extremity constituted by amino acid at position (-42) to the extremity constituted by amino acid at position (-1) represented on fig. 4a and fig. 4b,
- the one extending from the extremity constituted by amino acid at position (-29) to the extremity constituted by amino acid at position (-1) represented on fig. 4a and fig. 4b.

Other advantageous recombinant polypeptides of the invention consist in one of the following amino acid sequences:

- the one extending from the extremity constituted by amino acid at position (-43) to the extremity constituted by amino acid at position (-1) represented on fig. 5,
- the one extending from the extremity constituted by amino acid at position (-30) to the extremity constituted by amino acid at position (-1) represented on fig. 5.

In eukaryotic cells, these polypeptides can be used as signal peptides, the role of which is to initiate the translocation of a protein from its site of synthesis, but which is excised during translocation.

Oth r advantageous peptides of th invention consist in one of the following amino acid sequence:

- the one extending from the extremity constituted by amino acid at position (12) to the extremity constituted by amino acid at position (31) represented on fig. 3a and fig. 3b, or
- the one extending from the extremity constituted by amino acid at position (36) to the extremity constituted by amino acid at position (55) represented on fig. 3a and fig. 3b, or
- the one extending from the extremity constituted by amino acid at position (77) to the extremity constituted by amino acid at position (96) represented on fig. 3a and fig. 3b, or
- the one extending from the extremity constituted by amino acid at position (101) to the extremity constituted by amino acid at position (120) represented on fig. 3a and fig. 3b, or
- the one extending from the extremity constituted by amino acid at position (175) to the extremity constituted by amino acid at position (194) represented on fig. 3a and fig. 3b, or
- the one extending from the extremity constituted by amino acid at position (211) to the extremity constituted by amino acid at position (230) represented on fig. 3a and fig. 3b, or
- the one extending from the extremity constituted by amino acid at position (275) to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b.

Other advantageous peptides of the invention consist in one of the following amino acid sequence:

- the one extending from the extremity constituted by amino acid at position (12) to the extremity constituted by amino acid at position (31) represented n fig. 4a and fig. 4b, r

- the one extending from the xtremity constituted by amino acid at position (36) to the xtremity constituted by amino acid at position (55) repr sent d on fig. 4a and fig. 4b, or
- the one extending from the extremity constituted by amino acid at position (77) to the extremity constituted by amino acid at position (96) represented on fig. 4a and fig. 4b, or
- the one extending from the extremity constituted by amino acid at position (101) to the extremity constituted by amino acid at position (120) represented on fig. 4a and fig. 4b, or
- the one extending from the extremity constituted by amino acid at position (175) to the extremity constituted by amino acid at position (194) represented on fig. 4a and fig. 4b, or
- the one extending from the extremity constituted by amino acid at position (211) to the extremity constituted by amino acid at position (230) represented on fig. 4a and fig. 4b, or
- the one extending from the extremity constituted by amino acid at position (275) to the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b.

Other advantageous peptides of the invention consist in one of the following amino acid sequence:
- the one extending from the extremity constituted by amino acid at position (12) to the extremity constituted by amino acid at position (31) represented on fig. 5, or
- the one extending from the extremity constituted by amino acid at position (36) to the extremity constituted by amino acid at position (55) represented on fig. 5, or
- the one extending from the extremity constituted by amino acid at position (77) to th extremity

constituted by amino acid at position (96) represent d on fig. 5, or

- the one extending from the extremity constituted by amino acid at position (101) to the extremity constituted by amino acid at position (120) represented on fig. 5, or

- the one extending from the extremity constituted by amino acid at position (175) to the extremity constituted by amino acid at position (194) represented on fig. 5, or

- the one extending from the extremity constituted by amino acid at position (211) to the extremity constituted by amino acid at position (230) represented on fig. 5, or

- the one extending from the extremity constituted by amino acid at position (275) to the extremity constituted by amino acid at position (295) represented on fig. 5.

It is to be noted that the above mentioned polypeptides are derived from the expression products of a DNA derived from the nucleotide sequence coding for a protein of 32-kDa secreted by Mycobacterium tuberculosis as explained hereafter in the examples.

The invention also relates to the amino acid sequences constituted by the above mentioned polypeptides and a protein or an heterologous sequence with respect to said polypeptide, said protein or heterologous sequence comprising for instance from about 1 to about 1000 amino acids. These amino acid sequences will be called fusion proteins.

In an advantageous fusion protein of the invention, the heterologous protein is β-galactosidase.

Other advantageous fusion proteins of the invention are the ones containing an heterologous protein resulting fr m th expression f one of th following plasmids:

pEX1
pEX2
pEX3
pUEX1 pmTNF MPH
pUEX2
pUEX3

The invention also relates to any nucleotide sequence coding for a polypeptide of the invention.

The invention also relates to nucleic acids comprising nucleotide sequences which hybridize with the nucleotide sequences coding for any of the above mentioned polypeptides under the following hybridization conditions:

- hybridization and wash medium: 3 X SSC, 20% formamide (1 X SSC is 0,15 M NaCl, 0.015 M sodium citrate, pH 7.0),
- hybridization temperature (HT) and wash temperature (WT) for the nucleic acids of the invention defined by x-y: i.e. by the sequence extending from the extremity constituted by the nucleotide at position (x) to the extremity constituted by the nucleotide at position (y) represented on fig. 3a and fig. 3b.

1 - 182	HT = WT = 69°C
1 - 194	HT = WT = 69°C
1 - 212	HT = WT = 69°C
1 - 218	HT = WT = 69°C
1 - 272	HT = WT = 69°C
1 - 359	HT = WT = 71°C
1 - 1241	HT = WT = 73°C
1 - 1358	HT = WT = 73°C
183 - 359	HT = WT = 70°C
183 - 1241	HT = WT = 73°C
183 - 1358	HT = WT = 73°C
195 - 359	HT = WT = 70°C
195 - 1241	HT = WT = 73°C
195 - 1358	HT = WT = 73°C
213 - 359	HT = WT = 70°C

213 - 1241	HT = WT = 73°C
213 - 1358	HT = WT = 73°C
219 - 359	HT = WT = 71°C
219 - 1241	HT = WT = 73°C
219 - 1358	HT = WT = 73°C
234 - 359	HT = WT = 71°C
234 - 1241	HT = WT = 74°C
234 - 1358	HT = WT = 73°C
273 - 359	HT = WT = 71°C
273 - 1241	HT = WT = 74°C
273 - 1358	HT = WT = 73°C
360 - 1241	HT = WT = 73°C
360 - 1358	HT = WT = 73°C
1242 - 1358	HT = WT = 62°C

The above mentioned temperatures are to be considered as approximately ± 5°C.

The invention also relates to nucleic acids comprising nucleotide sequences which are complementary to the nucleotide sequences coding for any of the above mentioned polypeptides.

It is to be noted that in the above defined nucleic acids, as well as in the hereafter defined nucleic acids, the nucleotide sequences which are brought into play are such that T can be replaced by U.

A group of preferred nucleic acids of the invention comprises one at least of the following nucleotide sequences:

- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (182) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (273) to the extremity constituted by nucleotide at position (359) represented in fig. 3a and fig. 3b,

- the one extending from the extremity constituted by nucl otide at position (360) to th extremity constituted by nucleotide at position (1241) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (1242) to the extremity constituted by nucleotide at position (1358), wherein N represents one of the five A, T, C, G or I nucleotides, represented in fig. 3a and fig. 3b,

or above said nucleotide sequences wherein T is replaced by U,

or nucleic acids which hybridize with said above mentioned nucleotide sequences or the complementary sequences thereof.

A group of preferred nucleic acids of the invention comprises one at least of the following nucleotide sequences:

- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (182) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (273) to the extremity constituted by nucleotide at position (359) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (360) to the extremity constituted by nucleotide at position (1241) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (1242) to the extremity constituted by nucleotide at position (1358), wherein N represents one of the five A, T, C, G or I nucleotides, represented in fig. 4a and fig. 4b,

or above said nucleotide sequences wherein T is r placed by U,

or nucleic acids which hybridiz with said above mentioned nucleotid sequences r the complementary sequences thereof.

A group of preferred nucleic acids of the invention comprises one at least of the following nucleotide sequences:

- the one extending from the extremity constituted by nucleotide at position (130) to the extremity constituted by nucleotide at position (219) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (220) to the extremity constituted by nucleotide at position (1104) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (1104) to the extremity constituted by nucleotide at position (1299), wherein N represents one of the five A, T, C, G or I nucleotides, represented in fig. 5,

or above said nucleotide sequences wherein T is replaced by U,

or nucleic acids which hybridize with said above mentioned nucleotide sequences or the complementary sequences thereof.

Other preferred nucleic acids of the invention comprise one at least of the following nucleotide sequences:

- the one extending from the extremity constituted by nucleotide at position (195) to the extremity constituted by nucleotide at position (359) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (213) to the extremity constituted by nucleotide at position (359) represented in fig. 3a and fig. 3b,

- the one extending from the extremity constituted by nucleotide at position (219) to the extremity constituted by nucleotid at position (359) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (183) to the extremity constituted by nucleotide at position (359) represented in fig. 3a and fig. 3b.

Other preferred nucleic acids of the invention comprise one at least of the following nucleotide sequences:

- the one extending from the extremity constituted by nucleotide at position (195) to the extremity constituted by nucleotide at position (359) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (213) to the extremity constituted by nucleotide at position (359) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (219) to the extremity constituted by nucleotide at position (359) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (183) to the extremity constituted by nucleotide at position (359) represented in fig. 4a and fig. 4b.

Another preferred group of nucleic acids of the invention comprises the following nucleotide sequences:

- the one extending from the extremity constituted by nucleotide at position (360) to the extremity constituted by nucleotide at position (1358) represented in fig. 3a and fig. 3b.

Another preferred group of nucleic acids of the invention comprises the following nucleotide sequences:

- the one extending from th extremity constituted by nucleotide at position (360) to the extr mity constituted by nucl otid at position (1358) represented in fig. 4a and fig. 4b.

According to another advantageous embodiment, nucleic acids of the invention comprises one of the following sequences:

- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (194) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (212) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (218) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (272) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (359) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (1358) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (1241) represented in fig. 3a and fig. 3b,

- the one extending from th extremity constituted by nucl otide at position (183) to the xtremity constituted by nucleotid at position (1358) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (183) to the extremity constituted by nucleotide at position (1241) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (195) to the extremity constituted by nucleotide at position (1358) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (195) to the extremity constituted by nucleotide at position (1241) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (213) to the extremity constituted by nucleotide at position (1358) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (213) to the extremity constituted by nucleotide at position (1241) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (219) to the extremity constituted by nucleotide at position (1358) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (219) to the extremity constituted by nucleotide at position (1241) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (234) to the extremity constituted by nucleotide at position (1358) represented in fig. 3a and fig. 3b,

- the one extending from the extremity constituted by nucleotide at position (234) to the extremity constituted by nucleotide at position (1241) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (273) to the extremity constituted by nucleotide at position (1241) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (273) to the extremity constituted by nucleotide at position (1358) represented in fig. 3a and fig. 3b.

According to another advantageous embodiment, nucleic acids of the invention comprises one of the following sequences:

- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (194) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (212) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (218) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (272) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (359) represented in fig. 4a and fig. 4b,

- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotid at position (1358) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (1241) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (183) to the extremity constituted by nucleotide at position (1358) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (183) to the extremity constituted by nucleotide at position (1241) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (195) to the extremity constituted by nucleotide at position (1358) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (195) to the extremity constituted by nucleotide at position (1241) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (213) to the extremity constituted by nucleotide at position (1358) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (213) to the extremity constituted by nucleotide at position (1241) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (219) to the extremity constituted by nucleotide at position (1358) represented in fig. 4a and fig. 4b,

- the one extending from the extremity constituted by nucleotide at position (219) to the xtremity constituted by nucleotid at position (1241) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (234) to the extremity constituted by nucleotide at position (1358) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (234) to the extremity constituted by nucleotide at position (1241) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (273) to the extremity constituted by nucleotide at position (1241) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (273) to the extremity constituted by nucleotide at position (1358) represented in fig. 4a and fig. 4b.

Preferred nucleic acids of the invention consist in one of the following nucleotide sequences:

- the one extending from the extremity constituted by nucleotide at position (183) to the extremity constituted by nucleotide at position (359) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (195) to the extremity constituted by nucleotide at position (359) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (213) to the extremity constituted by nucleotide at position (359) represented in fig. 3a and fig. 3b,
- th on extending from the extremity constituted by nucleotid at position (219) t the extremity

constituted by nucleotide at position (359) represented in fig. 3a and fig. 3b,

- the one extending from th extremity constituted by nucleotide at position (234) to the extremity constituted by nucleotide at position (359) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (273) to the extremity constituted by nucleotide at position (359) represented in fig. 3a and fig. 3b.

Preferred nucleic acids of the invention consist in one of the following nucleotide sequences:

- the one extending from the extremity constituted by nucleotide at position (183) to the extremity constituted by nucleotide at position (359) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (195) to the extremity constituted by nucleotide at position (359) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (213) to the extremity constituted by nucleotide at position (359) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (219) to the extremity constituted by nucleotide at position (359) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (234) to the extremity constituted by nucleotide at position (359) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (273) to the extremity constituted by nucleotide at position (359) represented in fig. 4a and fig. 4b.

These nucleotide sequence can be us d as nucleotid signal sequences, coding for the corresponding signal peptide.

Preferred nucleic acids of the invention consist in one of the following nucleotide sequences:

- the one extending from the extremity constituted by nucleotide at position (360) to the extremity constituted by nucleotide at position (1241) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (360) to the extremity constituted by nucleotide at position (1358) represented in fig. 3a and fig. 3b.

Preferred nucleic acids of the invention consist in one of the following nucleotide sequences:

- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (182) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (194) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (212) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (218) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (272) represented in fig. 3a and fig. 3b,

- the one extending from the extremity constituted by nucl otide at position (1) to the extremity constituted by nucleotide at position (359) repres nted in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (1241) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (1358) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (183) to the extremity constituted by nucleotide at position (1241) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (183) to the extremity constituted by nucleotide at position (1358) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (195) to the extremity constituted by nucleotide at position (1241) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (195) to the extremity constituted by nucleotide at position (1358) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (213) to the extremity constituted by nucleotide at position (1241) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (213) to the extremity constituted by nucleotide at position (1358) repr sented in fig. 3a and fig. 3b,

- the one extending from the extremity constituted by nucleotide at position (219) to th xtr mity constituted by nucleotide at position (1241) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (219) to the extremity constituted by nucleotide at position (1358) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (234) to the extremity constituted by nucleotide at position (1241) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (234) to the extremity constituted by nucleotide at position (1358) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (273) to the extremity constituted by nucleotide at position (1241) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (273) to the extremity constituted by nucleotide at position (1358) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (1242) to the extremity constituted by nucleotide at position (1358) represented in fig. 3a and fig. 3b.

Preferred nucleic acids of the invention consist in one of the following nucleotide sequences:

- the one extending from the extremity constituted by nucleotide at position (360) to the extremity constituted by nucleotide at position (1241) represented in fig. 4a and fig. 4b,
- th one xtending from the extremity constituted by nucleotide at position (360) to the extremity

constituted by nucleotide at position (1358) repres nted in fig. 4a and fig. 4b.

Preferred nucleic acids of the invention consist in one of the following nucleotide sequences:

- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (182) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (194) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (212) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (218) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (272) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (359) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (1241) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (1358) represented in fig. 4a and fig. 4b,

- the one ext nding from the extremity c nstituted by nucleotide at position (183) to the extremity constituted by nucleotide at position (1241) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (183) to the extremity constituted by nucleotide at position (1358) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (195) to the extremity constituted by nucleotide at position (1241) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (195) to the extremity constituted by nucleotide at position (1358) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (213) to the extremity constituted by nucleotide at position (1241) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (213) to the extremity constituted by nucleotide at position (1358) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (219) to the extremity constituted by nucleotide at position (1241) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (219) to the extremity constituted by nucleotide at position (1358) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (234) to the extremity constitut d by nucleotide at position (1241) represented in fig. 4a and fig. 4b,

- the one extending from the extremity constituted by nucleotide at position (234) to the extremity constituted by nucleotide at position (1358) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (273) to the extremity constituted by nucleotide at position (1241) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (273) to the extremity constituted by nucleotide at position (1358) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (1242) to the extremity constituted by nucleotide at position (1358) represented in fig. 4a and fig. 4b.

Preferred nucleic acids of the invention consist in one of the following nucleotide sequences:

- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (129) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (219) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (1104) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (1299) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (90) to the extremity constituted by nucleotide at position (219) represented in fig. 5,
- the one extending from the extremity constituted by nucleotid at position (90) to the extremity

constituted by nucl otide at position (1299) r pr s nt d in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (90) to the extremity constituted by nucleotide at position (1104) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (130) to the extremity constituted by nucleotide at position (1104) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (130) to the extremity constituted by nucleotide at position (1299) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (220) to the extremity constituted by nucleotide at position (1299) represented in fig. 5.

Preferred nucleic acids of the invention consist in one of the following nucleotide sequences:
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (129) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (219) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (1104) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (1299) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (90) to the extremity constituted by nucleotide at position (219) represented in fig. 5,

- the one extending from the extremity constituted by nucleotide at position (90) to the extremity constituted by nucleotid at position (1104) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (90) to the extremity constituted by nucleotide at position (1299) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (130) to the extremity constituted by nucleotide at position (219) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (130) to the extremity constituted by nucleotide at position (1104) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (130) to the extremity constituted by nucleotide at position (1299) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (220) to the extremity constituted by nucleotide at position (1104) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (220) to the extremity constituted by nucleotide at position (1299) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (1104) to the extremity constituted by nucleotide at position (1299) represented in fig. 5.

The invention also relates to any recombinant nucleic acids containing at least a nucleic acid of the invention inserted in an heterologous nucleic acid.

The invention relates more particularly to recombinant nucleic acid such as d fined, in which the nucleotide sequence of the invention is preceded by a promoter (particularly an inducible promoter) under the control of which the transcription of said sequence is liable to be processed and possibly followed by a sequence coding for transcription termination signals.

The invention also relates to the recombinant nucleic acids in which the nucleic acid sequences coding for the polypeptide of the invention and possibly the signal peptide, are recombined with control elements which are heterologous with respect to the ones to which they are normally associated within the bacteria gene and, more particularly, the regulation elements adapted to control their expression in the cellular host which has been chosen for their production.

The invention also relates to recombinant vectors, particularly for cloning and/or expression, comprising a vector sequence, notably of the type plasmid, cosmid or phage, and a recombinant nucleic acid of the invention, in one of the non essential sites for its replication.

Appropriate vectors for expression of the recombinant antigen are the following one:

pEX1 pmTNF MPH
pEX2 pIGRI
pEX3
pUEX1
pUEX2
pUEX3

The pEX1, pEX2 and pEX3 vectors are commercially available and can be obtained from Boehringer Mannheim.

The pUEX1, pUEX2 and pUEX3 vectors are also commercially available and can be obtained from Amersham.

According to an advantageous embodiment of th invention, the recombinant vector contains, in one of its non essential sites for its replication, necessary elements to promote the expression of polypeptides according to the invention in a cellular host and possibly a promoter recognized by the polymerase of the cellular host, particularly an inducible promoter and possibly a signal sequence and/or an anchor sequence.

According to another additional embodiment of the invention, the recombinant vector contains the elements enabling the expression by E. coli of a nucleic acid according to the invention inserted in the vector, and particularly the elements enabling the expression of the gene or part thereof of β-galactosidase.

The invention also relates to a cellular host which is transformed by a recombinant vector according to the invention, and comprising the regulation elements enabling the expression of the nucleotide sequence coding for the polypeptide according to the invention in this host.

The invention also relates to a cellular host chosen from among bacteria such as E. coli, transformed by a vector as above defined, and defined hereafter in the examples, or chosen from among eukaryotic organism, such as CHO cells, insect cells, Sf9 cells [Spodoptera frugiperda] infected by the virus Ac NPV (Autographa californica nuclear polyhydrosis virus) containing suitable vectors such as pAc 373 pYM1 or pVC3, BmN [Bombyx mori] infected by the virus BmNPV containing suitable vectors such as pBE520 or p89B310.

The invention relates to an expression product of a nucleic acid expressed by a transformed cellular host according to the invention.

The invention also relates to nucleotidic probes, hybridizing with anyone of the nucleic acids or with their complementary sequences,

and particularly the probes chosen among the following nucleotidic sequ nces gathered in Table 1, and represented in fig. 9.

TABLE 1

Probes A(i), A(ii), A(iii), A(iv) and A(v)

A(i) CAGCTTGTTGACAGGGTTCGTGGC
A(ii) GGTTCGTGGCGCCGTCACG
A(iii) CGTCGCGCGCCTAGTGTCGG
A(iv) CGGCGCCGTCGGTGGCACGGCGA
A(v) CGTCGGCGCGGCCCTAGTGTCGG

Probe B

TCGCCCGCCCTGTACCTG

Probe C

GCGCTGACGCTGGCGATCTATC

Probe D

CCGCTGTTGAACGTCGGGAAG

Probe E

AAGCCGTCGGATCTGGGTGGCAAC

Probes F(i), F(ii), F(iii) and F(iv)

F(i) ACGGCACTGGGTGCCACGCCCAAC
F(ii) ACGCCCAACACCGGGCCCGCCGCA
F(iii) ACGGGCACTGGGTGCCACGCCCAAC
F(iv) ACGCCCCAACACCGGGCCCGCGCCCCA

or their complementary nucleotidic sequences.

The hybridization conditions can be the following ones:

- hybridization and wash medium: 3 X SSC, 20% formamide (1 X SSC is 0,15 M NaCl, 0.015 M sodium citrat ,

pH 7.0),
- hybridization temperature (HT) and wash temperature (WT):

(WT) °C:	HT and WT (°C)
A(i)	50
A(ii)	50
A(iii)	52
A(iv)	60
A(v)	52
B	48
C	50
D	45
E	52
F(i)	55
F(ii)	59
F(iii)	55
F(iv)	59

These probes might enable to differentiate M. tuberculosis from other bacterial strains and in particular from the following mycobacteria species:
- Mycobacterium marinum, Mycobacterium scrofulaceum, Mycobacterium gordonae, Mycobacterium szulgai, Mycobacterium intracellulare, Mycobacterium xenopi, Mycobacterium gastri, Mycobacterium nonchromogenicum, Mycobacterium terrae and Mycobacterium triviale, and more particularly from M. bovis, Mycobacterium kansasii, Mycobacterium avium, Mycobacterium phlei and Mycobacterium fortuitum.

The invention also relates to DNA or RNA primers which can be used for the synthesis of nucleotidic sequences according to the invention by PCR (polymerase chain reaction technique), such as described in US Patents n° 4,683,202 and n° 4,683,195 and European Patent n° 200362.

The invention also relates to any DNA or RNA primer constituted by about 15 to about 25 nucle tides

of a nucleotide sequence coding for a polypeptide according to the invention.

The invention also relates to any DNA or RNA primer constituted by about 15 to about 25 nucleotides liable to hybridize with a nucleotide sequence coding for a polypeptide according to the invention.

The invention also relates to any DNA or RNA primer constituted by about 15 to about 25 nucleotides complementary to a nucleotide sequence coding for a polypeptide according to the invention.

The sequences which can be used as primers are given in Table 2 hereafter (sequences P1 to P6 or their complement) and illustrated in fig. 9 :

TABLE 2

P1	GAGTACCTGCAGGTGCCGTCGCCGTCGATGGGCCG
P2	ATCAACACCCCGGCGTTCGAGTGGTAC
P2 compl.	GTACCACTCGAACGCCGGGGTGTTGAT
P3	TGCCAGACTTACAAGTGGGA
P3 compl.	TCCCACTTGTAAGTCTGGCA
P4	TCCTGACCAGCGAGCTGCCG
P4 compl.	CGGCAGCTCGCTGGTCAGGA
P5	CCTGATCGGCCTGGCGATGGGTGACGC
P5 compl.	GCGTCACCCATCGCCAGGCCGATCAGG
P6 compl.	GCGCCCCAGTACTCCCAGCTGTGCGT

compl. = complement

The sequences can be combined in twelve different primer-sets (given in Table 3) which allow enzymatical amplification by the polymerase chain reaction (PCR) technique of any of the nucleotide sequences of the invention, and more particularly the one extending from the extremity constituted by nucleotide at position 1 to the extremity constituted by nucleotide at position 1358, as well as the nucleotide sequence of antigen α of BCG (17).

The detection of the PCR amplified product can be achieved by a hybridization reaction with an oligonucleotide s quence of at least 10 nucleotides which is located between PCR primers which have been used to amplify the DNA.

The PCR products of the nucleotide sequences of the invention can be distinguished from the α-antigen gene of BCG or part thereof by hybridization techniques (dot-spot, Southern blotting, etc.) with the probes indicated in Table 3. The sequences of these probes can be found in Table 1 hereabove.

TABLE 3

Primer set	Detection with probe
1. P1 and the complement of P2	B
2. P1 and the complement of P3	B
3. P1 and the complement of P4	B
4. P1 and the complement of P5	B or C
5. P1 and the complement of P6	B, C, D or E
6. P2 and the complement of P5	C
7. P2 and the complement of P6	C, D or E
8. P3 and the complement of P5	C
9. P3 and the complement of P6	C, D or E
10. P4 and the complement of P5	C
11. P4 and the complement of P6	C, D or E
12. P5 and the complement of P6	D or E

It is to be noted that enzymatic amplification can also be achieved with all oligonucleotides with sequences of about 15 consecutive bases of the primers given in Table 2. Primers with elongation at the 5'-end or with a small degree of mismatch may not considerably affect the outcome of the enzymatic amplification if the mismatches do not interfere with the base-pairing at the 3'-end of the primers.

Specific enzymatic amplification of the nucleotid sequences of th invention and not of the BCG gene can be achieved when the probes (given in Table 1) or their complements are used as amplification primers.

When the above mentioned probes of Table 1 are used as primers, the primer sets are constituted by any of the nucleotide sequences (A, B, C, D, E, F) of Table 1 in association with the complement of any other nucleotide sequence, chosen from A, B, C, D, E or F, it being understood that sequence A means any of the sequences A(i), A(ii), A(iii), A(iv), A(v) and sequence F, any of the sequences F(i), F(ii), F(iii) and F(iv).

Advantageous primer sets for enzymatic amplification of the nucleotide sequence of the invention can be one of the following primer sets given in Table 3bis hereafter:

<u>TABLE 3BIS</u>

A(i) or A(ii) or A(iii) or A(iv) or A(v)	and the complement of B
A(i) or A(ii) or A(iii) or A(iv) or A(v)	and the complement of C
B	and the complement of C
A(i) or A(ii) or A(iii) or A(iv) or A(v)	and the complement of F

A(i) or A(ii) or A(iii) or A(iv) or A(v)	and the complement of D
A(i) or A(ii) or A(iii) or A(iv) or A(v)	and the complement of E
B	and the complement of D
B	and the complement of E
B	and the complement of F
C	and the complement of D
C	and the complement of E
C	and the complement of F
D	and the complement of E
D	and the complement of F
E	and the complement of F

A(i), A(ii), A(iii), A(iv), A(v), B, C, D, E and F having the nucleotide sequence indicated in Table 1.

In the case of amplification of a nucleotide sequence of the invention with any of the above mentioned primer sets defined in Table 3bis hereabove, the detection of the amplified nucleotide sequence can be achieved by a hybridization reaction with an oligonucleotide sequence of at least 10 nucleotides, said sequence being located between the PCR primers which have been used to amplify the nucleotide sequence. An oligonucleotide sequence located between said two primers can be determined from figure 9 where the primers A, B, C, D, E and F are represented by the boxed sequences respectively named probe region A, probe region B, probe region C, probe region D, probe region E and probe region F.

The invention also relates to a kit for enzymatic amplification of a nucleotid sequence by PCR technique and detection of the amplified nucleotide sequence containing
- one of the PCR primer sets defined in Table 3 and one of the detection probes of the invention, advantageously the probes defined in Table 1,
or one of the PCR primer sets defined in Table 3bis, and a detection sequence consisting for instance in an oligonucleotide sequence of at least 10 nucleotides, said sequence being located (fig. 9) between the two PCR primers constituting the primer set which has been used for amplifying said nucleotide sequence.

The invention also relates to a process for preparing a polypeptide according to the invention comprising the following steps:
- the culture in an appropriate medium of a cellular host which has previously been transformed by an appropriate vector containing a nucleic acid according to the invention,
- the recovery of the polypeptide produced by the above said transformed cellular host from the above said culture medium, and
- the purification of the polypeptide produced, eventually by means of immobilized metal ion affinity chromatography (IMAC).

The polypeptides of the invention can be prepared according to the classical techniques in the field of peptide synthesis.

The synthesis can be carried out in homogeneous solution or in solid phase.

For instance, the synthesis technique in homogeneous solution which can be used is the one described by Houbenweyl in the book titled "Methode der rganischen ch mie" (Method of organic chemistry)

edited by E. Wunsh, vol. 15-I et II. THIEME, Stuttgart 1974.

The polypeptid s f the invention can also be prepared according to the method described by R.D. MERRIFIELD in the article titled "Solid phase peptide synthesis" (J.P. Ham.Socks. , 45, 2149-2154).

The invention also relates to a process for preparing the nucleic acids according to the invention.

A suitable method for chemically preparing the single-stranded nucleic acids (containing at most 100 nucleotides of the invention) comprises the following steps :

- DNA synthesis using the automatic β-cyanoethyl phosphoramidite method described in Bioorganic Chemistry 4; 274-325, 1986.

In the case of single-stranded DNA, the material which is obtained at the end of the DNA synthesis can be used as such.

A suitable method for chemically preparing the double-stranded nucleic acids (containing at most 100 bp of the invention) comprises the following steps:

- DNA synthesis of one sense oligonucleotide using the automatic β-cyanoethyl phosphoramidite method described in Bioorganic Chemistry 4; 274-325, 1986, and DNA synthesis of one anti-sense oligonucleotide using said above-mentioned automatic β-cyanoethyl phosphoramidite method,

- combining the sense and anti-sense oligonucleotides by hybridization in order to form a DNA duplex,

- cloning the DNA duplex obtained into a suitable plasmid vector and recovery of the DNA according to classical methods, such as restriction enzyme digestion and agarose gel electrophoresis.

A method for the chemical preparation of nucleic acids of length greater than 100 nucleotides - or bp,

in the case of double-stranded nucleic acids - comprises the following steps :

- assembling of chemically synthesized oligonucleotides, provided at their ends with different restriction sites, the sequences of which are compatible with the succession of amino acids in the natural peptide, according to the principle described in Proc. Nat. Acad. Sci. USA 80; 7461-7465, 1983,

- cloning the DNA thereby obtained into a suitable plasmid vector and recovery of the desired nucleic acid according to classical methods, such as restriction enzyme digestion and agarose gel electrophoresis.

The invention also relates to antibodies themselves formed against the polypeptides according to the invention.

It goes without saying that this production is not limited to polyclonal antibodies.

It also relates to any monoclonal antibody produced by any hybridoma liable to be formed according to classical methods from splenic cells of an animal, particularly of a mouse or rat, immunized against the purified polypeptide of the invention on the one hand, and of cells of a myeloma cell line on the other hand, and to be selected by its ability to produce the monoclonal antibodies recognizing the polypeptide which has been initially used for the immunization of the animals.

The invention also relates to any antibody of the invention labeled by an appropriate label of the enzymatic, fluorescent or radioactive type.

The peptides which are advantageously used to produce antibodies, particularly monoclonal antibodies, are the following ones gathered in Table 4:

TABLE 4a (see fig. 4a and 4b)

Amino acid position (NH_2-terminal)		Amino acid position (COOH-terminal)
12	QVPSPSMGRDIKVQFQSGGA	31
36	LYLLDGLRAQDDFSGWDINT	55
77	SFYSDWYQPACRKAGCQTYK	96
101	LTSELPGWLQANRHVKPTGS	120
175	KASDMWGPKEDPAWQRNDPL	194
211	CGNGKPSDLGGNNLPAKFLE	230
275	KPDLQRHWVPRPTPGPPQGA	294

TABLE 4b (see fig. 5)

Amino acid position (NH_2-terminal)		Amino acid position (COOH-terminal)
77	SFYSDWYQPACGKAGCQTYK	96
276	PDLQRALGATPNTGPAPQGA	295

The amino acid sequences are given in the 1-letter code.

Variations of the peptides listed in Table 4 are also possible depending on their intended use. For example, if the peptides are to be used to raise antisera, the peptides may be synthesized with an extra cysteine residue added. This extra cysteine residue is preferably added to the amino terminus and facilitates the coupling of the peptide to a carrier protein which is necessary to render the small peptide immunogenic. If the peptide is to be labeled for use in radioimmune assays, it may be advantageous to synthesize the protein with a tyrosine attached to either the amino or carboxyl t rminus to facilitate iodination. These

peptides possess therefore the primary sequence of th peptides listed in Table 4 but with additional amino acids which do not appear in the primary sequenc of the protein and whose sole function is to confer the desired chemical properties to the peptides.

The invention also relates to a process for detecting in vitro antibodies related to tuberculosis in a human biological sample liable to contain them, this process comprising

- contacting the biological sample with a polypeptide or a peptide according to the invention under conditions enabling an in vitro immunological reaction between said polypeptide and the antibodies which are possibly present in the biological sample and
- the in vitro detection of the antigen/antibody complex which may be formed.

Preferably, the biological medium is constituted by a human serum.

The detection can be carried out according to any classical process.

By way of example a preferred method brings into play an immunoenzymatic process according to ELISA technique or immunofluorescent or radioimmunological (RIA) or the equivalent ones.

Thus the invention also relates to any polypeptide according to the invention labeled by an appropriate label of the enzymatic, fluorescent, radioactive... type.

Such a method for detecting in vitro antibodies related to tuberculosis comprises for instance the following steps:

- deposit of determined amounts of a polypeptidic composition according to the invention in the wells of a titration microplate,
- introducti n into said w lls of increasing dilutions f the serum to be diagnosed,

- incubation of the microplate,
- repeated rinsing of the microplate,
- introduction into the wells of the microplate f labeled antibodies against the blood immunoglobulins,
- the labeling of these antibodies being carried out by means of an enzyme which is selected from among the ones which are able to hydrolyze a substrate by modifying the absorption of the radiation of this latter at least at a given wave length,
- detection by comparing with a control standard of the amount of hydrolyzed substrate.

The invention also relates to a process for detecting and identifying in vitro antigens of M. tuberculosis in a human biological sample liable to contain them, this process comprising:

- contacting the biological sample with an appropriate antibody of the invention under conditions enabling an in vitro immunological reaction between said antibody and the antigens of M. tuberculosis which are possibly present in the biological sample and the in vitro detection of the antigen/antibody complex which may be formed.

Preferably, the biological medium is constituted by sputum, pleural effusion liquid, broncho-alveolar washing liquid, urine, biopsy or autopsy material.

Appropriate antibodies are advantageously monoclonal antibodies directed against the peptides which have been mentioned in Table 4.

The invention also relates to an additional method for the in vitro diagnostic of tuberculosis in a patient liable to be infected by Mycobacterium tuberculosis comprising the following steps:

- the possible previous amplification of the amount of the nucleotide sequences according to the invention, liable to be contained in a biological sample taken

from said patient by means of a DNA primer set as above defined,
- contacting the above mentioned biological sample with a nucleotide probe of the invention, under conditions enabling the production of an hybridization complex formed between said probe and said nucleotide sequence,
- detecting the above said hybridization complex which has possibly been formed.

To carry out the in vitro diagnostic method for tuberculosis in a patient liable to be infected by Mycobacterium tuberculosis as above defined, the following necessary or kit can be used, said necessary or kit comprising:
- a determined amount of a nucleotide probe of the invention,
- advantageously the appropriate medium for creating an hybridization reaction between the sequence to be detected and the above mentioned probe,
- advantageously, reagents enabling the detection of the hybridization complex which has been formed between the nucleotide sequence and the probe during the hybridization reaction.

The invention also relates to an additional method for the in vitro diagnostic of tuberculosis in a patient liable to be infected by Mycobacterium tuberculosis comprising :
- contacting a biological sample taken from a patient with a polypeptide or a peptide of the invention, under conditions enabling an in vitro immunological reaction between said polypeptide or peptide and the antibodies which are possibly present in the biological sample and
- the in vitro detection of the antigen/antibody complex which has possibly been formed.

To carry out the in vitro diagnostic method for tuberculosis in a patient liable to be infected by

Mycobacterium tuberculosis, the following necessary or kit can be used, said necessary or kit comprising:

- a polypeptide or a p ptide according to the invention,
- reagents for making a medium appropriate for the immunological reaction to occur,
- reagents enabling to detect the antigen/antibody complex which has been produced by the immunological reaction, said reagents possibly having a label, or being liable to be recognized by a labeled reagent,

more particularly in the case where the above mentioned polypeptide or peptide is not labeled.

The invention also relates to an additional method for the _in vitro_ diagnostic of tuberculosis in a patient liable to be infected by _M. tuberculosis_, comprising the following steps:

- contacting the biological sample with an appropriate antibody of the invention under conditions enabling an _in vitro_ immunological reaction between said antibody and the antigens of _M. tuberculosis_ which are possibly present in the biological sample and - the _in vitro_ detection of the antigen/antibody complex which may be formed.

Appropriate antibodies are advantageously monoclonal antibodies directed against the peptides which have been mentioned in Table 4.

To carry out the _in vitro_ diagnostic method for tuberculosis in a patient liable to be infected by Mycobacterium tuberculosis, the following necessary or kit can be used, said necessary or kit comprising:

- an antibody of the invention,
- reagents for making a medium appropriate for the immunological reaction to occur,
- reagents enabling to detect the antigen/antibody complexes which have been produced by the immunological reaction, said reagent possibly having a label or being

liable to be recognized by a label reagent, more particularly in the case where the above mention d antibody is not labeled.

An advantageous kit for the diagnostic <u>in vitro</u> of tuberculosis comprises:

- at least a suitable solid phase system, e.g. a microtiter-plate for deposition thereon of the biological sample to be diagnosed <u>in vitro</u>,
- a preparation containing one of the monoclonal antibodies of the invention,
- a specific detection system for said monoclonal antibody,
- appropriate buffer solutions for carrying out the immunological reaction between a test sample and said monoclonal antibody on the one hand, and the bonded monoclonal antibodies and the detection system on the other hand.

The invention also relates to a kit, as described above, also containing a preparation of one of the polypeptides or peptides of the invention, said antigen of the invention being either a standard (for quantitative determination of the antigen of <u>M. tuberculosis</u> which is sought) or a competitor, with respect to the antigen which is sought, for the kit to be used in a competition dosage process.

The invention also relates to an immunogenic composition comprising a polypeptide or a peptide according to the invention, in association with a pharmaceutically acceptable vehicle.

The invention also relates to a vaccine composition comprising among other immunogenic principles anyone of the polypeptides or peptides of the invention or the expression product of the invention, possibly coupled to a natural protein or to a synth tic polypeptide having a sufficient molecular weight so that the conjugat is able to induce <u>in vivo</u>

the production of antibodies neutralizing Mycobacterium tuberculosis, or induce in vivo a cellular immune response by activating M. tuberculosis antigen-responsive T cells.

The peptides of the invention which are advantageously used as immunogenic principle have one of the following sequences:

TABLE 4a (see fig. 4a and 4b)

Amino acid position (NH_2-terminal)		Amino acid position (COOH-terminal)
12	QVPSPSMGRDIKVQFQSGGA	31
36	LYLLDGLRAQDDFSGWDINT	55
77	SFYSDWYQPACRKAGCQTYK	96
101	LTSELPGWLQANRHVKPTGS	120
175	KASDMWGPKEDPAWQRNDPL	194
211	CGNGKPSDLGGNNLPAKFLE	230
275	KPDLQRHWVPRPTPGPPQGA	294

TABLE 4b (see fig. 5)

Amino acid position (NH_2-terminal)		Amino acid position (COOH-terminal)
77	SFYSDWYQPACGKAGCQTYK	96
276	PDLQRALGATPNTGPAPQGA	299

The amino acid sequences are given in the 1-letter code.

Other characteristics and advantages of the invention will appear in the following examples and the figures illustrating the invention.

Figures 1(A) and 1(B) correspond to the identification of six purified λgt11 M. tuberculosis recombinant clones. Figur 1(A) corresponds to the

EcoRI restriction analysis of clone 15, clone 16, clone 17, clone 19, clone 24 and EcoRI-HindIII digested lambda DNA-molecular weight marker lane (in kilobase pairs) (M) (Boehringer).

Figure 1(B) corresponds to the immunoblotting analysis of crude lysates of E. coli lysogenized with clone 15, clone 16, clone 17, clone 19, clone 23 and clone 24.

Arrow (<—) indicates fusion protein produced by recombinant λgt11-M-tuberculosis clones. Expression and immunoblotting were as described above. Molecular weight (indicated in kDa) were estimated by comparison with molecular weight marker (High molecular weight-SDS calibration kit, Pharmacia).

Figure 2 corresponds to the restriction map of the DNA inserts in the λgt11 M. tuberculosis recombinant clones 17 and 24 identified with polyclonal anti-32-kDa (BCG) antiserum as above defined and of clones By1, By2 and By5 selected by hybridization with a 120 bp EcoRI-Kpn I restriction fragment of clone 17.

DNA was isolated from λgt11 phage stocks by using the Lambda Sorb Phage Immunoadsorbent, as described by the manufacturer (Promega). Restriction sites were located as described above. Some restriction sites (*) were deduced from a computer analysis of the nucleotide sequence.

The short vertical bars (|——|) represent linker derived EcoRI sites surrounding the DNA inserts of recombinant clones. The lower part represents a magnification of the DNA region containing the antigen of molecular weight of 32-kDa, that has been sequenced. Arrows indicate strategies and direction of dideoxy-sequencing. (——>) fragment subcloned in Bluescribe M13; (<——>) fragment subcloned in mp10 and mp11 M13 vectors; (■——>) sequence det rmined with the use of a synth tic oligonucleotid .

Figures 3a and 3b correspond to the nucl otide and amino acid sequences of th general formula of the antigens of the invention.

Figures 4a and 4b correspond to the nucleotide and amino acid sequences of one of the antigens of the invention.

Two groups of sequences resembling the E. coli consensus promoter sequences are boxed and the homology to the consensus is indicated by italic bold letters. Roman bold letters represent a putative Shine-Dalgarno motif.

The NH_2-terminal amino acid sequence of the mature protein which is underlined with a double line happens to be very homologous - 29/32 amino acids - with the one of MPB 59 antigen (34). Five additional ATG codons, upstream of the ATG at position 273 are shown (dotted underlined). Vertical arrows (⇓) indicate the presumed NH_2 end of clone 17 and clone 24. The option taken here arbitrarily represents the 59 amino acid signal peptide corresponding to ATG_{183}.

Figure 5 corresponds to the nucleotide and amino acid sequences of the antigen of 32-kDa of the invention.

The NH_2-terminal amino acid sequence of the mature protein which is underlined with a double line happens to be very homologous - 29/32 amino acids - with the one of MPB 59 antigen (34). Vertical arrows (⇓) indicate the presumed NH_2 end of clone 17 and clone 24.

Figure 6 is the hydropathy pattern of the antigen of the invention of a molecular weight of 32-kDa and of the antigen α of BCG (17).

Figure 7 represents the homology between the amino acid sequences of the antigen of 32-kDa of the invention and of antigen α of BCG (revised version).

Identical amino acids; (:) evolutionarily conserved replacem nt f an amino acid (.), and absence

of homology () are indicated. Underlined sequence (=) represents th signal peptide, th opti n taken here arbitrarily representing the 43-amino acid signal peptide corresponding to ATG_{91}. Dashes in the sequences indicate breaks necessary for obtaining the optimal alignment.

Figure 8 illustrates the fact that the protein of 32-kDa of the invention is selectively recognized by human tuberculous sera.

Figure 8 represents the immunoblotting with human tuberculous sera, and anti-β-galactosidase monoclonal antibody. Lanes 1 to 6: E. coli lysate expressing fusion protein (140 kDa); lanes 7 to 12:unfused β-galactosidase (114 kDa). The DNA insert of clone 17 (2.7 kb) was subcloned into $pUEX_2$ and expression of fusion protein was induced as described by Bresson and Stanley (4). Lanes 1 and 7 were probed with the anti-β-galactosidase monoclonal antibody: lanes 4, 5, 6 and 10, 11, 12 with 3 different human tuberculous sera highly responding towards purified protein of the invention of 32-kDa; lanes 2 and 3 and 8 and 9 were probed with 2 different low responding sera.

Figure 9 represents the nucleic acid sequence alignment of the 32-kDa protein gene of M. tuberculosis of the invention (upper line), corresponding to the sequence in fig. 5, of the gene of fig. 4a and 4b of the invention (middle line), and of the gene for antigen α of BCG (lower line).

Dashes in the sequence indicate breaks necessary for obtaining optimal alignment of the nucleic acid sequence.

The primer regions for enzymatical amplification are boxed (P1 to P6).

The specific probe regions are boxed and respectively defined by probe region A, probe region B,

probe region C, probe region D, probe region E and probe r gion F.

It is t be noted that the numbering of nucleotides is different from the numbering of figures 3a and figure 3b, and of figure 5, because nucleotide at position 1 (on figure 9) corresponds to nucleotide 234 on Figure 3a, and corresponds to nucleotide 91 on figure 5.

Figure 10a corresponds to the restriction and genetic map of the pIGRI plasmid used in Example IV for the expression of the P_{32} antigen of the invention in E. coli.

On this figure, underlined restriction sites are unique.

Figure 10b corresponds to the pIGRI nucleic acid sequence.

On this figure, the origin of nucleotide stretches used to construct plasmid pIGRI are specified hereafter.

Position	
3422-206 :	lambda PL containing EcoRI blunt-MboII blunt fragment of pPL(λ) (Pharmacia)
207-384 :	synthetic DNA sequence
228-230 :	initiation codon ATG of first cistron
234-305 :	DNA encoding amino acids 2 to 25 of mature mouse TNF
306-308 :	stop codon (TAA) first cistron
311-312 :	initiation codon (ATG) second cistron
385-890 :	$rrnBT_1T_2$ containing HindIII-SspI fragment from pKK223 (Pharmacia)
891-3421 :	DraI-EcoRI blunt fragment of pAT_{153} (Bioexcellence) containing the

tetracycline resistance gen and the origin of replication.

Table 5 hereafter corresponds to the complete restriction site analysis of pIGRI.

Table 5

===

****
* RESTRICTION-SITE ANALYSIS *
****

Name of the plasmid : pIGRI

Total number of bases is: 3423.
Analysis done on the complete sequence.

List of cuts by enzyme.

Acc I :	370	2765									
Acy I :	735	2211	2868	2982	3003						
Afl III :	1645										
Aha III :	222										
Alu I :	386	1088	1345	1481	1707	2329	2732	3388	3403		
Alw NI :	1236										
Apa LI :	1331										
Asp 718I :	208										
Asu I :	329	494	623	713	1935	1977	2156	2280	2529	2617	289
	3244										
Ava I :	1990										
Ava II :	329	494	1935	1977	2280	2529	2617				
Bal I :	1973										
Bam HI :	3040										
Bbe I :	2214	2871	2985	3006							
Bbv I :	389	1316	1735	1753	1866	1869	2813	3202			
Bbv I* :	1017	1223	1226	1973	1997	2630					
Bbv II :	1822	2685									
Bgl I :	2253	2487									
Bin I :	15	903	1001	1087	3048						
Bin I* :	902	999	2313	3035							

Table 5 (Con't)

Bsp HI	:	855	925	2926								
Bsp MI	:	382	2361									
Bst NI	:	213	475	585	753	1486	1499	1620	1975	2358	3287	
Cau II	:	4	683	716	1268	1933	2159	2883	3247			
Cfr 10I	:	2132	2486	2646	3005	3014	3255					
Cfr I	:	1971	2476	2884	3016	3120						
Cla I	:	3393										
Cvi JI	:	190	263	270	380	386	391	421	607	625	714	77
		791	1088	1117	1160	1171	1236	1315	1340	1345	1481	157
		1605	1623	1634	1707	1726	1926	1931	1973	2010	2092	213
		2157	2162	2300	2310	2329	2370	2427	2435	2465	2478	249
		2544	2588	2732	2748	2804	2822	2886	2894	2932	2946	301
		3087	3122	3245	3269	3388	3403					
Cvi QI	:	209	3253									
Dde I	:	133	336	343	518	608	664	962	1371	1835		
Dpn I	:	9	236	897	909	987	995	1006	1081	1957	2274	228
		2320	2592	2951	3042	3069						
Dra II	:	1935	1977	2892								
Dra III	:	293										
Dsa I	:	309	1968	2887								
Eco 31I	:	562										
Eco 47III	:	341	1773	2642	2923	3185						
Eco 57I	:	214										
Eco 57I*	:	1103										
Eco 78I	:	2212	2869	2983	3004							
Eco NI	:	196	2792									
Eco RII	:	211	473	583	751	1484	1497	1618	1973	2356	3285	
Eco RV	:	3232										
Fnu 4HI	:	378	479	1031	1237	1240	1305	1448	1603	1721	1724	174
		1855	1858	1987	2001	2008	2011	2130	2209	2254	2311	239
		2479	2644	2695	2802	2836	2839	3117	3120	3191		
Fnu DII	:	489	1021	1602	1784	1881	2003	2029	2174	2184	2313	237
		2440	2445	2472	2601	2716	3072					
Fok I	:	415	799	3317								
Fok I*	:	763	2370	2415	3269							
Gsu I	:	339	2035									
Gsu I*	:	2589										

Table 5 (Con't)

Hae I :	775	791	1171	1623	1634	1973	2370	2427	2499		
Hae II :	343	541	1405	1775	2214	2644	2871	2925	2985	3006	318
Hae III :	625	714	775	791	1171	1605	1623	1634	1973	2157	237
	2427	2478	2499	2588	2822	2886	2894	3018	3122	3245	
Hga I :	158	181	743	2035	2185	2776					
Hga I* :	955	1533	2429	2461	3015						
Hgi AI :	139	1335	1954	2245	2832	3143					
Hgi CI :	208	2126	2210	2649	2867	2981	3002	3296	3339		
Hgi JII :	2934	2948									
Hha I :	342	489	540	1021	1130	1304	1404	1471	1741	1774	196
	2000	2062	2213	2472	2603	2643	2718	2870	2924	2984	300
	3158	3186	3318								
Hin P1I :	340	487	538	1019	1128	1302	1402	1469	1739	1772	196
	1998	2060	2211	2470	2601	2641	2716	2868	2922	2982	300
	3156	3184	3316								
Hind II :	107	371	2766								
Hind III :	384	3386									
Hinf I :	367	1275	1671	1746	1891	2112	2410	2564	2784		
Hpa II :	3	682	716	1077	1267	1293	1440	1932	2133	2159	239
	2487	2647	2723	2883	3006	3015	3030	3247	3256		
Hph I :	94	138	181	663	914	1900	2121	2975	3020	3302	
Hph I* :	6										
Kpn I :	212										
Mae I :	364	899	1152	1928	3187						
Mae II :	274	698	944	1847	1871	2460	2516				
Mae III :	169	255	304	313	1109	1225	1288	2267	2534	3202	329
Mbo I :	7	234	895	907	985	993	1004	1079	1955	2272	228
	2318	2590	2949	3040	3067						
Mbo II :	207	422	917	1779	1827	2419	2690				
Mbo II* :	988	2944									
Mme I* :	1252	1436	3112	3199							
Mnl I :	1218	1542	1948	2446	2630						
Mnl I* :	208	289	337	711	1467	1750	2116	2143	2181	2242	254
	2811	3030	3234	3294							
Mse I :	179	186	221	433	764	941	3361	3383	3420		
Mst I :	1963	2061	3157								
Nae I :	2134	2488	2648	3016							

Table 5 (Con't)

Nar I :	2211	2868	2982	3003							
Nco I :	309										
Nhe I :	3186										
Nla III :	166	230	313	512	567	859	929	1649	1828	1962	216
	2226	2241	2369	2486	2672	2711	2857	2930	3068	3415	
Nla IV :	210	330	496	1578	1617	1936	1979	2093	2128	2163	221
	2530	2651	2869	2893	2983	3004	3042	3088	3298	3341	
Nru I :	2445										
Nsp BII :	1062	1307	2278								
Nsp HI :	1649	2857									
Pfl MI :	293	2052	2101								
Ple I :	375	1754									
Ple I* :	1269	2778									
Ppu MI :	1935	1977									
Pss I :	1938	1980	2895								
Pst I :	379										
Rsa I :	210	3254									
Sal I :	369	2764									
Scr FI :	4	213	475	585	683	716	753	1268	1486	1499	162
	1933	1975	2159	2358	2883	3247	3287				
Sdu I :	139	1335	1954	2245	2832	2934	2948	3143			
Sec I :	3	309	1485	1968	2046	2248	2881	2887	3286	3300	
Sfa NI :	597	765	2392	2767	3178	3291					
Sfa NI* :	1548	1985	2380	3001	3013	3202					
Sph I :	2857										
Sso II :	2	211	473	583	681	714	751	1266	1484	1497	161
	1931	1973	2157	2356	2881	3245	3285				
Sty I :	309	2046									
Taq I :	252	370	613	1547	2149	2290	2765	3078	3393		
Taq IIB :	1749										
Taq IIB* :	2751										
Tth111II :	38	1054									
Tth111II* :	633	1022	1061								
Xba I :	363										
Xho II :	7	895	907	993	1004	3040					
Xma III :	2476										
Xmn I :	414										

Total number of cuts is : 705.

Table 5 (Con't)

Sorted list of enzymes by n° of cuts.

Cvi JI	61	Sdu I	8	Tth111II*	3	Ava I	1
Fnu 4HI	31	Cau II	8	Nsp BII	3	Taq IIB	1
Hha I	25	Bbv I	8	Fok I	3	Alw NI	1
Hin PlI	25	Mbo II	7	Pfl MI	3	Dra III	1
Hae III	21	Ava II	7	Hind II	3	Afl III	1
Nla IV	21	Mae II	7	Dsa I	3	Cla I	1
Nla III	21	Sfa NI	6	Bsp HI	3	Eco 57I*	1
Hpa II	20	Xho II	6	Pss I	3	Nhe I	1
Scr FI	18	Hgi AI	6	Mst I	3	Gsu I*	1
Sso II	18	Sfa NI*	6	Hgi JII	2	Bal I	1
Fnu DII	17	Bbv I*	6	Ple I	2	Eco RV	1
Mbo I	16	Cfr 10I	6	Mbo II*	2	Sph I	1
Dpn I	16	Hga I	6	Cvi QI	2	Xma III	1
Mnl I*	15	Acy I	5	Acc I	2	Hph I*	1
Asu I	12	Bin I	5	Bgl I	2	Taq IIB*	1
Hae II	11	Cfr I	5	Ple I*	2	Eco 57I	1
Mae III	11	Hga I*	5	Gsu I	2	Kpn I	1
Hph I	10	Mae I	5	Ppu MI	2	Xba I	1
Bst NI	10	Eco 47III	5	Tth111II	2	Aha III	1
Eco RII	10	Mnl I	5	Hind III	2	Nru I	1
Sec I	10	Mme I*	4	Nsp HI	2	Bam HI	1
Dde I	9	Eco 78I	4	Rsa I	2	Apa LI	1
Hinf I	9	Nae I	4	Sal I	2	Asp 718I	1
Hae I	9	Bbe I	4	Bbv II	2	Eco 31I	1
Alu I	9	Bin I*	4	Bsp MI	2	Nco I	1
Hgi CI	9	Nar I	4	Sty I	2	Pst I	1
Mse I	9	Fok I*	4	Eco NI	2		
Taq I	9	Dra II	3	Xmn I	2		

List of non cutting selected enzymes.

Aat II , Afl II , Apa I , Asu II , Avr II , Bbv II* , Bcl I
Bgl II , Bsp MI* , Bsp MII , Bss HII , Bst EII , Bst XI , Eco 31I*
Eco RI , Esp I , Hpa I , Mlu I , Mme I , Nde I , Not I
Nsi I , Pma CI , Pvu I , Pvu II , Rsr II , Sac I , Sac II
Sau I , Sca I , Sci I , Sfi I , Sma I , Sna BI , Spe I
Spl I , Ssp I , Stu I , Taq IIA , Taq IIA* , Tth 111I , Vsp I
Xca I , Xho I , Xma I Total number of selected enzymes which do not cut: 45

Figure 11a corresponds to the restriction and genetic map of th pmTNF MPH plasmid used in Exampl V for the expression of the P_{32} antigen of the invention in E. coli.

Figure 11b corresponds to the pmTNF-MPH nucleic acid sequence.

On this figure, the origin of nucleotide stretches used to construct plasmid pmTNF-MPH is specified hereafter.

Position		
1-208 :	lambda PL containing EcoRI blunt-MboII blunt fragment of pPL(λ) (Pharmacia)	
209-436 :	synthetic DNA fragment	
230-232 :		initiation codon (ATG) of mTNF fusion protein
236-307 :		sequence encoding AA 2 to 25 of mature mouse TNF
308-384 :		multiple cloning site containing His_6 encoding sequence at position 315-332
385-436 :		HindIII fragment containing E. coli trp terminator
437-943 :	$rrnBT_1T_2$ containing HindIII-SspI fragment from pKK223 (Pharmacia)	
944-3474 :	DraI-EcoRI blunt fragment of pAT_{153} (Bioexcellence) containing the tetracycline resistance gene and the origin of replication.	

Table 6 hereafter corresponds to the complete restriction site analysis of pmTNF-MPH.

Table 6

==

****
* RESTRICTION-SITE ANALYSIS *
****

Done on DNA sequence PMTNFMPH.

Total number of bases is: 3474.
Analysis done on the complete sequence.

List of cuts by enzyme.
========================

Acc I	:	371	2818									
Acy I	:	788	2264	2921	3035	3056						
Afl II	:	387										
Afl III	:	1698										
Aha III	:	224										
Alu I	:	386	439	1141	1398	1534	1760	2382	2785	3441	3456	
Alw NI	:	1289										
Apa I	:	345										
Apa LI	:	1384										
Asp 718I	:	210										
Asu I	:	341	342	547	676	766	1988	2030	2209	2333	2582	267
		2945	3297									
Ava I	:	338	2043									
Ava II	:	547	1988	2030	2333	2582	2670					
Bal I	:	2026										
Bam HI	:	334	3093									
Bbe I	:	2267	2924	3038	3059							
Bbv I	:	1369	1788	1806	1919	1922	2866	3255				
Bbv I*	:	1070	1276	1279	2026	2050	2683					

Table 6 (Con't)

Bbv II	: 1875	2738									
Bgl I	: 2306	2540									
Bin I	: 17	342	956	1054	1140	3101					
Bin I*	: 329	955	1052	2366	3088						
Bsp HI	: 908	978	2979								
Bsp MI	: 2414										
Bsp MII	: 354										
Bst NI	: 215	528	638	806	1539	1552	1673	2028	2411	3340	
Cau II	: 6	339	340	736	769	1321	1986	2212	2936	3300	
Cfr 10I	: 374	2185	2539	2699	3058	3067	3308				
Cfr I	: 2024	2529	2937	3069	3173						
Cla I	: 3446										
Cvi JI	: 192	265	272	343	350	361	386	400	439	444	47
	660	678	767	828	844	1141	1170	1213	1224	1289	136
	1393	1398	1534	1632	1658	1676	1687	1760	1779	1979	198
	2026	2063	2145	2189	2210	2215	2353	2363	2382	2423	248
	2488	2518	2531	2552	2597	2641	2785	2801	2857	2875	293
	2947	2985	2999	3071	3140	3175	3298	3322	3441	3456	
Cvi QI	: 211	3306									
Dde I	: 135	571	661	717	1015	1424	1888				
Dpn I	: 11	238	336	950	962	1040	1048	1059	1134	2010	232
	2342	2373	2645	3004	3095	3122					
Dra II	: 1988	2030	2945								
Dra III	: 295	331									
Dsa I	: 345	2021	2940								
Eco 31I	: 615										
Eco 47III	: 1826	2695	2976	3238							
Eco 57I	: 216										
Eco 57I*	: 1156										
Eco 78I	: 2265	2922	3036	3057							
Eco NI	: 198	2845									
Eco RI	: 309										
Eco RII	: 213	526	636	804	1537	1550	1671	2026	2409	3338	
Eco RV	: 3285										

Table 6 (con't)

Fnu 4HI :	401	417	532	1084	1290	1293	1358	1501	1656	1774	177
	1795	1908	1911	2040	2054	2061	2064	2183	2262	2307	236
	2447	2532	2697	2748	2855	2889	2892	3170	3173	3244	
Fnu DII :	542	1074	1655	1837	1934	2056	2082	2227	2237	2366	243
	2493	2498	2525	2654	2769	3125					
Fok I :	468	852	3370								
Fok I* :	816	2423	2468	3322							
Gsu I :	2088										
Gsu I* :	2642										
Hae I :	361	828	844	1224	1676	1687	2026	2423	2480	2552	
Hae II :	594	1458	1828	2267	2697	2924	2978	3038	3059	3240	
Hae III :	343	361	678	767	828	844	1224	1658	1676	1687	202
	2210	2423	2480	2531	2552	2641	2875	2939	2947	3071	317
	3298										
Hga I :	160	183	796	2088	2238	2829					
Hga I* :	1008	1586	2482	2514	3068						
Hgi AI :	141	1388	2007	2298	2885	3196					
Hgi CI :	210	2179	2263	2702	2920	3034	3055	3349	3392		
Hgi JII :	345	2987	3001								
Hha I :	542	593	1074	1183	1357	1457	1524	1794	1827	2017	205
	2115	2266	2525	2656	2696	2771	2923	2977	3037	3058	321
	3239	3371									
Hin P1I :	540	591	1072	1181	1355	1455	1522	1792	1825	2015	205
	2113	2264	2523	2654	2694	2769	2921	2975	3035	3056	320
	3237	3369									
Hind II :	109	372	2819								
Hind III :	384	437	3439								
Hinf I :	368	1328	1724	1799	1944	2165	2463	2617	2837		

Table 6 (Con't)

Hpa II :	5	339	355	375	735	769	1130	1320	1346	1493	198
	2186	2212	2450	2540	2700	2776	2936	3059	3068	3083	330
	3309										
Hph I :	96	140	183	716	967	1953	2174	3028	3073	3355	
Hph I* :	8	305	311	317							
Kpn I :	214										
Mae I :	365	952	1205	1981	3240						
Mae II :	276	330	751	997	1900	1924	2513	2569			
Mae III :	171	257	1162	1278	1341	2320	2587	3255	3343		
Mbo I :	9	236	334	948	960	1038	1046	1057	1132	2008	232
	2340	2371	2643	3002	3093	3120					
Mbo II :	209	475	970	1832	1880	2472	2743				
Mbo II* :	1041	2997									
Mme I* :	1305	1489	3165	3252							
Mnl I :	372	1271	1595	2001	2499	2683					
Mnl I* :	210	291	350	764	1520	1803	2169	2196	2234	2295	259
	2864	3083	3287	3347							
Mse I :	181	188	223	388	486	817	994	3414	3436		
Mst I :	2016	2114	3210								
Nae I :	2187	2541	2701	3069							
Nar I :	2264	2921	3035	3056							
Nco I :	345										
Nhe I :	3239										
Nla III :	168	232	349	382	565	620	912	982	1702	1881	201
	2222	2279	2294	2422	2539	2725	2764	2910	2983	3121	346
Nla IV :	212	336	343	549	1631	1670	1989	2032	2146	2181	221
	2265	2583	2704	2922	2946	3036	3057	3095	3141	3351	339
Nru I :	2498										
Nsp BII :	412	1115	1360	2331							
Nsp HI :	382	1702	2910								

Table 6 (Con't)

Pfl MI	:	295	2105	2154								
Ple I	:	376	1807									
Ple I*	:	1322	2831									
Pma CI	:	331										
Ppu MI	:	1988	2030									
Pss I	:	1991	2033	2948								
Rsa I	:	212	3307									
Sal I	:	370	2817									
Scr FI	:	6	215	339	340	528	638	736	769	806	1321	153
		1552	1673	1986	2028	2212	2411	2936	3300	3340		
Sdu I	:	141	345	1388	2007	2298	2885	2987	3001	3196		
Sec I	:	5	338	345	1538	2021	2099	2301	2934	2940	3339	335
Sfa NI	:	650	818	2445	2820	3231	3344					
Sfa NI*	:	420	1601	2038	2433	3054	3066	3255				
Sma I	:	340										
Sph I	:	382	2910									
Sso II	:	4	213	337	338	526	636	734	767	804	1319	153
		1550	1671	1984	2026	2210	2409	2934	3298	3338		
Stu I	:	361										
Sty I	:	345	2099									
Taq I	:	254	371	666	1600	2202	2343	2818	3131	3446		
Taq IIB	:	1802										
Taq IIB*	:	2804										
Tth111II	:	40	1107									
Tth111II*	:	686	1075	1114								
Xba I	:	364										
Xho II	:	9	334	948	960	1046	1057	3093				
Xma I	:	338										
Xma III	:	2529										
Xmn I	:	467										

Total number of cuts is : 743.

Table 6 (con't)

List of non cutting selected enzymes.

Aat II , Asu II , Avr II ; Bbv II* , Bcl I , Bgl II , Bsp MI*
Bss HII , Bst EII , Bst XI , Eco 31I* , Esp I , Hpa I , Mlu I
Mme I , Nde I , Not I , Nsi I , Pst I , Pvu I , Pvu II
Rsr II , Sac I , Sac II , Sau I , Sca I , Sci I , Sfi I
Sna BI , Spe I , Spl I , Ssp I , Taq IIA , Taq IIA* , Tth 111I
Vsp I , Xca I , Xho I

Total number of selected enzymes which do not cut: 38

Figure 12a corresponds to the restriction and genetic map of the plasmid pIG2 used to make the intermediary construct pIG2 Mt32 as describ d in Example IV for the subcloning of the P_{32} antigen in plasmid pIGRI.

Figure 12b corresponds to the pIG2 nucleic acid sequence.

On this figure, the origin of nucleotide stretches used to construct plasmid pIG2 is specified hereafter.

Position	
3300-206 :	lambda PL containing EcoRI-MboII blunt fragment of pPL(λ) (Pharmacia)
207-266 :	synthetic sequence containing multiple cloning site and ribosome binding site of which the ATG initiation codon is located at position 232-234
267-772 :	$rrnBT_1T_2$ containing HindIII-SspI fragment from pKK223 (Pharmacia)
773-3300 :	tetracycline resistance gene and origin of replication containing EcoRI-DraI fragment of pAT 153 (Bioexcellence)

Table 7 corresponds to the complete restriction site analysis of pIG2.

Table 7

```
*******************************
* RESTRICTION-SITE ANALYSIS *
*******************************
```

Done on DNA sequence pIG2

Total number of bases is: 3301.
Analysis done on the complete sequence.

List of cuts by enzyme.

Acc I :	252	2647									
Acy I :	617	2093	2750	2864	2885						
Afl III :	1527										
Aha III :	222										
Alu I :	268	970	1227	1363	1589	2211	2614	3270	3285		
Alw NI :	1118										
Apa LI :	1213										
Asp 718I :	208										
Asu I :	376	505	595	1817	1859	2038	2162	2411	2499	2774	312
Ava I :	1872										
Ava II :	376	1817	1859	2162	2411	2499					
Bal I :	1855										
Bam HI :	239	2922									
Bbe I :	2096	2753	2867	2888							
Bbv I :	271	1198	1617	1635	1748	1751	2695	3084			
Bbv I* :	899	1105	1108	1855	1879	2512					
Bbv II :	1704	2567									
Bgl I :	2135	2369									
Bin I :	15	247	785	883	969	2930					
Bin I* :	234	784	881	2195	2917						
Bsp HI :	737	807	2808								
Bsp MI :	264	2243									
Bst NI :	213	357	467	635	1368	1381	1502	1857	2240	3169	
Cau II :	4	565	598	1150	1815	2041	2765	3129			

Table 7 (Con't)

Cfr 10I :	2014	2368	2528	2887	2896	3137					
Cfr I :	1853	2358	2766	2898	3002						
Cla I :	3275										
Cvi JI :	190	262	268	273	303	489	507	596	657	673	97
	999	1042	1053	1118	1197	1222	1227	1363	1461	1487	150
	1516	1589	1608	1808	1813	1855	1892	1974	2018	2039	204
	2182	2192	2211	2252	2309	2317	2347	2360	2381	2426	247
	2614	2630	2686	2704	2768	2776	2814	2828	2900	2969	300
	3127	3151	3270	3285							
Cvi QI :	209	3135									
Dde I :	133	400	490	546	844	1253	1717				
Dpn I :	9	241	779	791	869	877	888	963	1839	2156	217
	2202	2474	2833	2924	2951						
Dra II :	1817	1859	2774								
Dsa I :	230	1850	2769								
Eco 31I :	444										
Eco 47III :	1655	2524	2805	3067							
Eco 57I :	214										
Eco 57I* :	985										
Eco 78I :	2094	2751	2865	2886							
Eco NI :	196	2674									
Eco RII :	211	355	465	633	1366	1379	1500	1855	2238	3167	
Eco RV :	3114										
Fnu 4HI :	260	361	913	1119	1122	1187	1330	1485	1603	1606	162
	1737	1740	1869	1883	1890	1893	2012	2091	2136	2193	227
	2361	2526	2577	2684	2718	2721	2999	3002	3073		
Fnu DII :	371	903	1484	1666	1763	1885	1911	2056	2066	2195	226
	2322	2327	2354	2483	2598	2954					
Fok I :	297	681	3199								
Fok I* :	645	2252	2297	3151							
Gsu I :	1917										
Gsu I* :	2471										
Hae I :	657	673	1053	1505	1516	1855	2252	2309	2381		
Hae II :	423	1287	1657	2096	2526	2753	2807	2867	2888	3069	
Hae III :	507	596	657	673	1053	1487	1505	1516	1855	2039	225
	2309	2360	2381	2470	2704	2768	2776	2900	3004	3127	
Hga I :	158	181	625	1917	2067	2658					

Table 7 (con't)

Hga I* :	837	1415	2311	2343	2897						
Hgi AI :	139	1217	1836	2127	2714	3025					
Hgi CI :	208	2008	2092	2531	2749	2863	2884	3178	3221		
Hgi JII :	2816	2830									
Hha I :	371	422	903	1012	1186	1286	1353	1623	1656	1846	188
	1944	2095	2354	2485	2525	2600	2752	2806	2866	2887	304
	3068	3200									
Hin P1I :	369	420	901	1010	1184	1284	1351	1621	1654	1844	188
	1942	2093	2352	2483	2523	2598	2750	2804	2864	2885	303
	3066	3198									
Hind II :	107	253	2648								
Hind III :	266	3268									
Hinf I :	249	1157	1553	1628	1773	1994	2292	2446	2666		
Hpa II :	3	564	598	959	1149	1175	1322	1814	2015	2041	227
	2369	2529	2605	2765	2888	2897	2912	3129	3138		
Hph I :	94	138	181	545	796	1782	2003	2857	2902	3184	
Hph I* :	6										
Kpn I :	212										
Mae I :	246	781	1034	1810	3069						
Mae II :	580	826	1729	1753	2342	2398					
Mae III :	169	991	1107	1170	2149	2416	3084	3172			
Mbo I :	7	239	777	789	867	875	886	961	1837	2154	216
	2200	2472	2831	2922	2949						
Mbo II :	207	304	799	1661	1709	2301	2572				
Mbo II* :	870	2826									
Mme I* :	1134	1318	2994	3081							
Mnl I :	253	1100	1424	1830	2328	2512					
Mnl I* :	208	593	1349	1632	1998	2025	2063	2124	2426	2693	291
	3116	3176									
Mse I :	179	186	221	315	646	823	3243	3265			
Mst I :	1845	1943	3039								
Nae I :	2016	2370	2530	2898							
Nar I :	2093	2750	2864	2885							
Nco I :	230										
Nhe I :	3068										
Nla III :	166	234	394	449	741	811	1531	1710	1844	2051	210
	2123	2251	2368	2554	2593	2739	2812	2950	3297		

Table 7 (Con't)

Nla IV :	210	241	378	1460	1499	1818	1861	1975	2010	2045	209
	2412	2533	2751	2775	2865	2886	2924	2970	3180	3223	
Nru I :	2327										
Nsp BII :	944	1189	2160								
Nsp HI :	1531	2739									
Pfl MI :	1934	1983									
Ple I :	257	1636									
Pl I* :	1151	2660									
Ppu MI :	1817	1859									
Pss I :	1820	1862	2777								
Pst I :	261										
Rsa I :	210	3136									
Sal I :	251	2646									
Scr FI :	4	213	357	467	565	598	635	1150	1368	1381	150
	1815	1857	2041	2240	2765	3129	3169				
Sdu I :	139	1217	1836	2127	2714	2816	2830	3025			
Sec I :	3	230	1367	1850	1928	2130	2763	2769	3168	3182	
Sfa NI :	479	647	2274	2649	3060	3173					
Sfa NI* :	1430	1867	2262	2883	2895	3084					
Sph I :	2739										
Sso II :	2	211	355	465	563	596	633	1148	1366	1379	150
	1813	1855	2039	2238	2763	3127	3167				
Ssp I :	226										
Sty I :	230	1928									
Taq I :	252	495	1429	2031	2172	2647	2960	3275			
Taq IIB :	1631										
Taq IIB* :	2633										
Tth111II :	38	936									
Tth111II* :	515	904	943								
Xba I :	245										
Xho II :	7	239	777	789	875	886	2922				
Xma III :	2358										
Xmn I :	296										
EcoRI :	3300										

T tal number of cuts is : 689.

Table 7 (con't)

List of non cutting selected enzymes.

Aat II	, Afl II	, Apa I	, Asu II	, Avr II	, Bbv II*	, Bcl I
Bgl II	, Bsp MI*	, Bsp MII	, Bss HII	, Bst EII	, Bst XI	, Dra III
Eco 31I*	, Esp I	, Hpa I	, Mlu I	, Mme I	, Nde I	, Not I
Nsi I	, Pma CI	, Pvu I	, Pvu II	, Rsr II	, Sac I	, Sac II
Sau I	, Sca I	, Sci I	, Sfi I	, Sma I	, Sna BI	, Spe I
Spl I	, Stu I	, Taq IIA	, Taq IIA*	, Tth 111I	, Vsp I	, Xca I
Xho I	, Xma I					

Total number of selected enzymes which do not cut: 44

Figure 13 corresponds to the amino acid sequence of the total fusion protein mTNF-His_6-P_{32}.

On this figure :

- the continuous underlined sequence (______) represents the mTNF sequence (first 25 amino acids),
- the dotted underlined sequence (-----) represents the polylinker sequence,
- the double underlined sequence (═════) represents the extra amino acids created at cloning site, and
- the amino acid marked with nothing is the antigen sequence starting from the amino acid at position 4 of figure 5.

Figure 14a and 14b correspond to the expression of the mTNF-His_6-P_{32} fusion protein in K12ΔH, given in Example VI, with Fig. 14a representing the Coomassie Brilliant Blue stained SDS-PAGE and 14b representing immunoblots of the gel with anti-32-kDa and anti-mTNF-antibody.

On fig. 14a, the lanes correspond to the following:

Lanes			
1.	protein molecular weight markers		
2.	pmTNF-MPH-Mt32	28°C	1 h induction
3.	"	42°C	"
4.	"	42°C	2 h induction
5.	"	42°C	3 h "
6.	"	28°C	4 h "
7.	"	42°C	4 h "
8.	"	28°C	5 h "
9.	"	42°C	5 h "

On fig. 14b, the lanes correspond to the following:

Lanes

1.	pmTNF-MPH-Mt32	28°C	1 h	induction
2.	"	42°C	1 h	"
3.	"	28°C	4 h	"
4.	"	42°C	4 h	"

Figure 15 corresponds to the IMAC elution profile of the recombinant antigen with decreasing pH as presented in Example VII.

Figure 16 corresponds to the IMAC elution profile of the recombinant antigen with increasing imidazole concentrations as presented in Example VII.

Figure 17 corresponds to the IMAC elution profile of the recombinant antigen with a step gradient of increasing imidazole concentrations as presented in Example VII.

EXAMPLE I:

MATERIAL AND METHODS

Screening of the λgt11 M. tuberculosis recombinant DNA library with anti-32-kDa antiserum

A λgt11 recombinant library constructed from genomic DNA of M. tuberculosis (Erdman strain), was obtained from R. Young (35). Screening was performed as described (14,35) with some modifications hereafter mentioned. λgt11 infected E. coli Y1090 (10^5 pfu per 150 mm plate) were seeded on NZYM plates (Gibco)(16) and incubated at 42°C for 24 hrs. To induce expression of the β-galactosidase-fusion proteins the plates were overlaid with isopropyl β-D-thiogalactoside (IPTG)-saturated filters (Hybond C extra, Amersham), and incubated for 2 hrs at 37°C. Screening was done with a polyclonal rabbit anti-32-kDa antiserum. Said polyclonal antiserum rabbit anti-32-kDa antiserum was obtained by raising antiserum against the P_{32} M. bovis BCG (strain 1173P2 - Institut Pasteur Paris) as follows: 400 μg (purified P_{32} protein of M. bovis BCG) per ml physiological saline were mixed with one volume

of incomplete Freund's adjuvant. The material was homogenized and inj cted intradermally in 50 μl doses, delivered at 10 sites in the back f the rabbits, at 0, 4, 7 and 8 weeks (adjuvant was replaced by the diluent for the last injection). One week later, the rabbits were bled and the sera tested for antibody level before being distributed in aliquots and stored at -80°C.

The polyclonal rabbit anti-32-kDa antiserum was pre-absorbed on E. coli lysate (14) and used at a final dilution of 1:300. A secondary alkaline-phosphatase anti-rabbit IgG conjugate (Promega), diluted at 1:5000 was used to detect the β-galactosidase fusion proteins. For color development nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were used. Reactive areas on the filter turned deep purple within 30 min. Usually three consecutive purification steps were performed to obtain pure clones. IPTG, BCIP and NBT were from Promega corp. (Madison WI.).

<u>Plaque screening by hybridization for obtaining the secondary clones BY1, By2 and By5 hereafter defined</u>

The procedure used was as described by Maniatis et al. (14).

<u>Preparation of crude lysates from λgt11 recombinant lysogens</u>

Colonies of E. coli Y1089 were lysogenized with appropriate λgt11 recombinants as described by Hyunh et al. (14). Single colonies of lysogenized E. coli Y1089 were inoculated into LB medium and grown to an optical density of 0.5 at 600nm at 30°C. After a heat shock at 45°C for 20 min., the production of β-galactosidase-fusion protein was induced by the addition of IPTG to a final concentration of 10 mM. Incubation was continued for 60 min. at 37°C and cells were quickly harvested by centrifugation. Cells were concentrated 50 times in buffer (10 mM Tris pH 8.0, 2 mM EDTA) and rapidly frozen into liquid nitrogen. The samples were lysed by

thawing and tr ated with 100 µg/ml DNas I in EcoRI restriction buffer, for 5-10 minutes at 37°C.

Immunoblotting (Western blotting) analysis:

After SDS-PAGE electrophoresis, recombinant lysogen proteins were blotted onto nitrocellulose membranes (Hybond C, Amersham) as described by Towbin et al. (29). The expression of mycobacterial antigens, fused to β-galactosidase in E. coli Y1089 was visualized by the binding of a polyclonal rabbit anti-32-kDa antiserum (1:1000) obtained as described in the above paragraph "Screening of the λgt11 M. tuberculosis recombinant DNA library with anti-32-kDa antiserum" and using a monoclonal anti-β-galactosidase antibody (Promega). A secondary alkaline-phosphatase anti-rabbit IgG conjugate (Promega) diluted at 1:5000, was used to detect the fusion proteins.

The use of these various antibodies enables to detect the β-galactosidase fusion protein. This reaction is due to the M. tuberculosis protein because of the fact that non fused-β-galactosidase is also present on the same gel and is not recognized by the serum from tuberculous patients.

In order to identify selective recognition of recombinant fusion proteins by human tuberculous sera, nitrocellulose sheets were incubated overnight with these sera (1:50)(after blocking aspecific protein binding sites). The human tuberculous sera were selected for their reactivity (high or low) against the purified 32-kDa antigen of M. bovis BCG tested in a Dot blot assay as previously described (31). Reactive areas on the nitrocellulose sheets were revealed by incubation with peroxidase conjugated goat anti-human IgG antibody (Dakopatts, Copenhagen, Denmark)(1:200) for 4 hrs and after repeated washings color reaction was dev lop d by adding p roxidas substrate (α-

chloronaphtol) (Bio-Rad) in th presence of peroxidase and hydrogen peroxide.

Recombinant DNA analysis

Initial identification of M. tuberculosis DNA inserts in purified λgt11 clones was performed by EcoRI restriction. After digestion, the excised inserts were run on agarose gels and submitted to Southern hybridization. Probes were labeled with α^{32}P-dCTP by random priming (10). Other restriction sites were located by single and double digestions of recombinant λgt11 phage DNA or their subcloned EcoRI fragments by HindIII, PstI, KpnI, AccI and SphI.

Sequencing

Sequence analysis was done by the primer extension dideoxy termination method of Sanger et al. (25) after subcloning of specific fragments in Bluescribe-M13 (6) or in mp10 and mp11 M13 vectors (Methods in Enzymology, vol. 101, 1983, p.20-89, Joachim Messing, New M13 vectors for cloning, Academic Press). Sequence analysis was greatly hampered by the high GC content of the M. tuberculosis DNA (65%). Sequencing reactions were therefore performed with several DNA polymerases: T7 DNA polymerase ("Sequenase" USB), Klenow fragment of DNA polymerase I (Amersham) and in some cases with AMV reverse transcriptase (Super RT, Anglian Biotechnology Ltd.) and sometimes with dITP instead of dGTP. Several oligodeoxynucleotides were synthesized and used to focus ambiguous regions of the sequence. The sequencing strategy is summarized in Fig. 2 In order to trace possible artefactual frameshifts in some ambiguous regions, a special program was used to define the most probable open reading frame in sequences containing a high proportion of GC (3). Several regions particularly prone to sequencing artefacts were confirmed or corrected by chemical sequencing (18). For this purpose, fragments were subcloned in the chemical

s quencing v ctor pGV462 (21) and analysed as described previously. S lected restriction fragments of about 250-350bp were isolated, made blunt-ended by treatment with either Klenow polymerase or Mung bean nuclease, and subcloned in the SmaI or HincII site of pGV462. Both strands of the inserted DNA were sequenced by single-end labeling at Tth 111I or BstEII (32) and a modified chemical degradation strategy (33).

Routine computer aided analysis of the nucleic acid and deduced amino acid sequences were performed with the LGBC program from Bellon (2). Homology searches used the FASTA programs from Pearson and Lipman (23) and the Protein Identification Resource (PIR) from the National Biomedical Research Fundation - Washington (NBRF)(NBRF/PIR data bank), release 16 (march 1988).

RESULTS

- <u>Screening of the λgt11M, M. tuberculosis recombinant DNA library with polyclonal anti-32-kDa antiserum</u> :

Ten filters representing $1.5x10^6$ plaques were probed with a polyclonal rabbit anti-32-kDa antiserum (8). Following purification, six independent positive clones were obtained.

<u>Analysis of recombinant clones</u>

EcoRI restriction analysis of these 6 purified λgt11 recombinant clones DNA, (Fig. 1A) revealed 4 different types of insert. Clone 15 had an insert with a total length of 3.8 kb with two additional internal EcoRI sites resulting in three DNA fragments of 1.8 kb, 1.5 kb and 0.5 kb. The DNA Insert of clone 16 was 1.7 kb long. Clones 17 and 19 had a DNA insert of almost identical length being 2.7 kb and
2.8 kb respectively.

Finally, clone 23 (not shown) and clone 24 both contained an insert of 4 kb with one additional EcoRI restriction site giving two fragments f 2.3 kb and

1.7 kb. Southern analysis (data not shown) showed that the DNA inserts of clones 15, 16, 19 and th small fragment (1.7 kb) of clone 24 only hybridized with themselves whereas clone 17 (2.7 kb) hybridized with itself but also equally well with the 2.3 kb DNA fragment of clone 24. Clones 15, 16 and 19 are thus distinct and unrelated to the 17, 23, 24 group. This interpretation was further confirmed by analysis of crude lysates of E. coli Y1089 lysogenized with the appropriate λgt11 recombinants and induced with IPTG. Western blot analysis (Fig. 1B), showed no fusion protein, either mature or incomplete, reactive with the polyclonal anti-32-kDa antiserum in cells expressing clones 15, 16 and 19. Clones 15, 16 and 19, were thus considered as false positives and were not further studied. On the contrary, cells lysogenized with clone 23 and 24 produced an immunoreactive fusion protein containing about 10 kDa of the 32-kDa protein. Clone 17 finally expressed a fusion protein of which the foreign polypeptide part is about 25 kDa long. The restriction endonuclease maps of the 2.3 kb insert of clone 24 and of the 2.7 kb fragment of clone 17 (Fig. 2) allowed us to align and orient the two inserts suggesting that the latter corresponds to a ±0.5 kb 5' extension of the first.

As clone 17 was incomplete, the same λgt11 recombinant M. tuberculosis DNA library was screened by hybridization with a 120 bp EcoRI-Kpnl restriction fragment corresponding to the very 5' end of the DNA insert of clone 17 (previously subcloned in a Blue Scribe vector commercialized by Vector cloning Systems (Stratagene Cloning System) (Fig.2). Three 5'-extended clones By1, By2 and By5 were isolated, analyzed by restriction and aligned. The largest insert, By5 contained the information for the entire coding region

(see below) flanked by 3.1 kb upstream and 1.1 kb downstream (Fig. 2).

DNA sequencing

The 1358 base pairs nucleotide sequence derived from the various λgt11 overlapping clones is represented in Fig. 3a and Fig. 3b. The DNA sequence contains a 1059 base pair open reading frame starting at position 183 and ending with a TAG codon at position 1242. It occurs that the NH_2-terminal amino-acid sequence, (phe-ser-arg-pro-gly-leu-pro-val-glu-tyr-leu-gln-val-pro-ser-pro-ser-met-gly-arg-asp-ile-lys-val-gln-phe-gln-ser-gly-gly-ala-asn) which can be located within this open reading frame from the nucleotide sequence beginning with a TTT codon at position 360 corresponds to the same NH_2-terminal amino acid sequence of the MPB 59 antigen except for the amino acids at position 20, 21, 31, which are respectively gly, cys and asn in the MPB 59 (34). Therefore, the DNA region upstream of this sequence is expected to encode a signal peptide required for the secretion of a protein of 32-kDa. The mature protein thus presumably consists of 295 amino acid residues from the N-terminal Phe (TTT codon) to the C-terminal Ala (GCC codon)(Fig. 5).

Six ATG codons were found to precede the TTT at position 360 in the same reading frame. Usage of any of these ATGs in the same reading frame would lead to the synthesis of signal peptides of 29,42,47,49,55 and 59 residues.

Hydropathy pattern

The hydropathy pattern coding sequence of the protein of 32-kDa of the invention and that of the antigen α of BCG (17) were determined by the method of Kyte and Doolittle (15). The nonapeptide profiles are shown in Fig. 6. Besides the initial hydrophobic signal peptide r gion, several hydrophilic domains could be

identified. It is interesting to note that the overall hydrophilicity pattern of the protein of 32-kDa of the invention is comparable to that of th BCG antigen α. For both proteins, a domain of highest hydrophilicity could be identified between amino acid residues 200 and 250.

Homology

Matsuo et al. (17) recently published the sequence of a 1095 nucleotide cloned DNA corresponding to the gene coding for the antigen α of BCG. The 978 bp coding region of M. bovis antigen α as revised in Infection and Immunity, vol. 58, p. 550-556, 1990, and 1017 bp coding regions of the protein of 32-kDa of the invention show a 77.5% homology, in an aligned region of 942 bp. At the amino acid level both precursor protein sequences share 75.6% identical residues. In addition, 17.6% of the amino acids correspond to evolutionary conserved replacements as defined in the algorithm used for the comparison (PAM250 matrix, ref 23). Figure 7 shows sequence divergences in the N-terminal of the signal peptide. The amino terminal sequence - 32 amino acids - of both mature proteins is identical except for position 31.

Human sera recognize the recombinant 32-kDa protein

Fig. 8 shows that serum samples from tuberculous patients when immunoblotted with a crude E. coli extract expressing clone 17 distinctly react with the 140 kDa fusion protein (lanes 4 to 6) contain the protein of 32-kDa of the invention, but not with unfused β-galactosidase expressed in a parallel extract (lanes 10 to 12). Serum samples from two negative controls selected as responding very little to the purified protein of 32-kDa of the invention does neither recognize the 140 kDa fused protein containing the protein of 32-kDa of the invention, nor the unfused β-galactosidase (lanes 2, 3 and 8 and 9). The 140 k-Da

fused protein and the unfused β-galactosidas w re easily localized reacting with the anti-β-galactosidas monoclonal antibody (lanes 1 to 7).

The invention has enabled to prepare a DNA region coding particularly for a protein of 32-kDa (cf. fig.5); said DNA region containing an open reading frame of 338 codons (stop codon non included). At position 220 a TTT encoding the first amino acid of the mature protein is followed by the 295 triplets coding for the mature protein of 32-kDa. The size of this open reading frame, the immunoreactivity of the derived fusion proteins, the presence of a signal peptide and, especially, the identification within this gene of a NH_2-terminal region highly homologous to that found in the MPB 59 antigen (31/32 amino acids homology) and in the BCG antigen α (31/32 amino acids homology)(see Fig. 7), strongly suggest that the DNA fragment described contains the complete cistron encoding the protein of 32-kDa secreted by M. tuberculosis, and which had never been so far identified in a non ambiguous way.

Six ATG codons were found to precede this TTT at position 220 in the same reading frame. Usage of any of these ATGs in the same reading frame would lead to the synthesis of signal peptides of 43, 48, 50, 56 or 60 residues. Among these various possibilities, initiation is more likely to take place either at ATG_{91} or ATG_{52} because both are preceded by a plausible E. coli-like promoter and a Shine-Dalgarno motif.

If initiation takes place at ATG_{91}, the corresponding signal peptide would code for a rather long peptide signal of 43 residues. This length however is not uncommon among secreted proteins from Gram positive bacteria (5). It would be preceded by a typical E. coli Shine-Dalgarno motif (4/6 residues homologous to AGGAGG) at a suitable distance.

If initiation takes place at ATG_{52}, the corresponding signal peptide would code for a peptide signal of 56 residues but would have a less stringent Shine-Dalgarno ribosome binding site sequence.

The region encompassing the translation termination triplet was particularly sensitive to secondary structure effects which lead to so-called compressions on the sequencing gels. In front of the TAG termination codon at position 1105, 22 out of 23 residues are G-C base pairs, of which 9 are G's.

Upstream ATG_{130}, a sequence resembling an E. coli promoter (11) comprising an hexanucleotide (TTGAGA) (homology 5/6 to TTGACA) and a AAGAAT box (homology 4/6 to TATAAT) separated by 16 nucleotides was observed. Upstream the potential initiating codon ATG_{91}, one could detect several sequences homologous to the E. coli "-35 hexanucleotide box", followed by a sequence resembling a TATAAT box. Among these, the most suggestive is illustrated on Fig. 3a and 3b. It comprises a TTGGCC at position 59 (fig. 3a and 3b)(homology 4/6 to TTGACA) separated by 14 nucleotides from a GATAAG (homology 4/6 to TATAAT). Interestingly this putative promoter region shares no extensive sequence homology with the promoter region described for the BCG protein α-gene (17) nor with that described for the 65 kDa protein gene (26, 28).

Searching the NBRF data bank (issue 16.0) any significant homology between the protein of 32-kDa of the invention and any other completely known protein sequence could not be detected. In particular no significant homology was observed between the 32-kDa protein and α and β subunits of the human fibronectin receptor (1). The NH_2-terminal sequence of the 32-kDa protein of the invention is highly homologous - 29/32 amino acids - to that previously published for BCG MPB 59 antigen (34) and to that of BCG α-antigen - 31/32

amino acids - (Matsuo, 17) and is identical in its first 6 amino acids with the 32-kDa protein of M. bovis BCG (9). However, the presumed initiating methionine precedes an additional 29 or 42 amino acid hydrophobic sequence which differs from the one of α-antigen (cf. Fig. 7), but displaying all the characteristics attributed to signal sequences of secreted polypeptides in prokaryotes (22).

Interestingly, no significant homology between the nucleic acid (1-1358) of the invention (cf. fig. 3a and 3b) and the DNA of the antigen α of Matsuo exists within their putative promoter regions.

EXAMPLE II: CONSTRUCTION OF A BACTERIAL PLASMID CONTAINING THE ENTIRE CODING SEQUENCE OF THE 32-kDa PROTEIN OF M. TUBERCULOSIS

In the previous example, in figure 2, the various overlapping λgt11 isolates covering the 32-kDa protein gene region from M. tuberculosis were described. Several DNA fragments were subcloned from these λgt11 phages in the Blue Scribe M13+ plasmid (Stratagene). Since none of these plasmids contained the entire coding sequence of the
32-kDa protein gene, a plasmid containing this sequence was reconstructed.

Step 1 : Preparation of the DNA fragments :

1) The plasmid BS-By5-800 obtained by subcloning HindIII fragments of By5 (cf. fig. 2) into the Blue Scribe M13⁺ plasmid (Stratagene), was digested with HindIII and a fragment of 800 bp was obtained and isolated from a 1% agarose gel by electroelution.

2) The plasmid BS-4.1 obtained by subcloning the 2,7 kb EcoRI insert from λgt11-17) into the Blue Scribe M13⁺ plasmid (Stratagene) (see fig.2 of patent application) was digested with HindIII and SphI and a fragment f 1500 bp was obtained and isolated from a 1% agarose gel by el ctroeluti n.

3) Blue Scribe M13$^+$ was digested with HindIII and SphI, and treated with calf intestine alkaline phosphatase (special quality for molecular biology, Boehringer Mannheim) as indicated by the manufacturer.

Step 2 : ligation :

The ligation reaction contained :

125 ng of the 800 bp HindIII fragment (1)

125 ng of the 1500 bp SphI-HindIII insert (2)

50 ng of the HindIII-SphI digested BSM13$^+$ vector (3)

2 µl of 10 ligation buffer (Maniatis et al., 1982)

1 µl of (= 2,5 U) of T4 DNA ligase (Amersham)

4 µl PEG 6000, 25% (w/v)

8 µl H_2O

The incubation was for 4 hours at 16°C.

Step 3 : Transformation :

100 µl of DH5α E. coli (Gibco BRL) were transformed with 10 µl of the ligation reaction (step 2) and plated on IPTG, X-Gal ampicillin plates, as indicated by the manufacturer. About 70 white colonies were obtained.

step 4 :

As the 800 bp fragment could have been inserted in both orientations, plasmidic DNA from several clones were analyzed by digestion with PstI in order to select one clone (different from clone 11), characterized by the presence of 2 small fragments of 229 and 294 bp. This construction contains the HindIII-HindIII-SphI complex in the correct orientation. The plasmid containing this new construction was called : "BS.BK.P_{32}.complet".

EXAMPLE III: EXPRESSION OF A POLYPEPTIDE OF THE INVENTION IN E. COLI:

The DNA sequence coding for a polypeptide, or part of it, can be linked to a ribosome binding site which is part of the expression vect r, r can b fused to

the information of another protein or peptide already present on the expression vector.

In the former case the information is expressed as such and hence devoid of any foreign sequences (except maybe for the aminoterminal methionine which is not always removed by E. coli).

In the latter case the expressed protein is a hybrid or a fusion protein.

The gene, coding for the polypeptide, and the expression vector are treated with the appropriate restriction enzyme(s) or manipulated otherwise as to create termini allowing ligation. The resulting recombinant vector is used to transform a host. The transformants are analyzed for the presence and proper orientation of the inserted gene. In addition, the cloning vector may be used to transform other strains of a chosen host. Various methods and materials for preparing recombinant vectors, transforming them to host cells and expressing polypeptides and proteins are described by Panayatatos, N., in "Plasmids, a practical approach (ed. K.G. Hardy, IRL Press) pp.163-176, by Old and Primrose, principals of gene manipulation (2d Ed, 1981) and are well known by those skilled in the art.

Various cloning vectors may be utilized for expression. Although a plasmid is preferable, the vector may be a bacteriophage or cosmid. The vector chosen should be compatible with the host cell chosen.

Moreover, the plasmid should have a phenotypic property that will enable the transformed host cells to be readily identified and separated from those which are not transformed. Such selection genes can be a gene providing resistance to an antibiotic like for instance, tetracyclin, carbenicillin, kanamycin, chloramphenicol, streptomycin, etc.

In order to expr ss th coding sequenc of a g ne in E. coli the expression vector should also contain

the necessary signals for transcription and translation.

Hence it should contain a promoter, synthetic or derived from a natural source, which is functional in E. coli. Preferably, although usually not absolutely necessary, the promoter should be controllable by the manipulator. Examples of widely used controllable promoters for expression in E. coli are the lac, the trp, the tac and the lambda PL and PR promoter.

Preferably, the expression vector should also contain a terminator of transcription functional in E. coli. Examples of used terminators of transcription are the trp and the rrnB terminators.

Furthermore, the expression vector should contain a ribosome binding site, synthetic or from a natural source, allowing translation and hence expression of a downstream coding sequence. Moreover, when expression devoid of foreign sequences is desired, a unique restriction site, positioned in such a way that it allows ligation of the sequence directly to the initiation codon of the ribosome binding site, should be present.

A suitable plasmid for performing this type of expression is pKK233-2 (Pharmacia). This plasmid contains the trc promoter, the lac Z ribosome binding site and the rrnB transcription terminator.

Also suitable is plasmid pIGRI (Innogenetics, Ghent, Belgium). This plasmid contains the tetracycline resistance gene and the origin of replication of pAT_{153} (available from Bioexcellence, Biores B.V., Woerden, The Netherlands), the lambda PL promoter up to the MboII site in the 5' untranslated region of the lambda N gene (originating from pPL(λ); Pharmacia).

Downstream from the PL promoter, a synthetic sequence was introduced which encodes a "two cistron" translation casette whereby the stop codon of the first

cistron (being the first 25 amino acids of TNF, except for Leu at position 1 which is converted to Val) is situated between the Shine-Dalgarno sequence and the initiation codon of the second ribosome binding site. The restriction and genetic map of pIGRI is represented in Fig. 10a.

Fig. 10b and Table 5 represent respectively the nucleic acid sequence and complete restriction site analysis of pIGRI.

However, when expression as a hybrid protein is desired, then the expression vector should also contain the coding sequence of a peptide or polypeptide which is (preferably highly) expressed by this vector in the appropriate host.

In this case the expression vector should contain a unique cleavage site for one or more restriction endonucleases downstream of the coding sequence.

Plasmids pEX1, 2 and 3 (Boehringer, Mannheim) and pUEX1, 2 and 2 (Amersham) are useful for this purpose.

They contain an ampicillin resistance gene and the origin of replication of pBR322 (Bolivar at al. (1977) Gene 2, 95-113), the lac Z gene fused at its 5' end to the lambda PR promoter together with the coding sequence for the 9 first amino acids of its natural gene cro, and a multiple cloning site at the 3' end of the lac Z coding sequence allowing production of a beta galactosidase fused polypeptide.

The pUEX vectors also contain the CI857 allele of the bacteriophage lambda CI repressor gene.

Also useful is plasmid pmTNF MPH (Innogenetics). It contains the tetracycline resistance gene and the origin of replication of pAT_{153} (obtainable from Bioexcellence, Biores B.V., Woerden. The Netherlands), the lambda PL promoter up to the MboII site in the N gen 5' untranslated region (originating from pPL(λ); Pharmacia), foll w d by a synth tic ribosom binding

site (see sequence data), and the information encoding the first 25 AA of mTNF (except for the initial Leu which is converted to Val). This sequence is, in turn, followed by a synthetic polylinker sequence which encodes six consecutive histidines followed by several proteolytic sites (a formic acid, CNBr, kallikrein, and E. coli protease VII sensitive site, respectively), each accessible via a different restriction enzyme which is unique for the plasmid (SmaI, NcoI, BspMII and StuI, respectively; see restriction and genetic map, Fig. 11a). Downstream from the polylinker, several transcription terminators are present including the E. coli trp terminator (synthetic) and the $rrnBT_1T_2$ (originating from pKK223-3; Pharmacia). The total nucleic acid sequence of this plasmid is represented in Fig. 11b.

Table 6 gives a complete restriction site analysis of pmTNF MPH.

The presence of 6 successive histidines allows purification of the fusion protein by Immobilized Metal Ion Affinity Chromatography (IMAC).

After purification, the foreign part of the hybrid protein can be removed by a suitable protein cleavage method and the cleaved product can then be separated from the uncleaved molecules using the same IMAC based purification procedure.

In all the above-mentioned plasmids where the lambda PL or PR promoter is used, the promoter is temperature-controlled by means of the expression of the lambda cI ts 857 allele which is either present on a defective prophage incorporated in the chromosome of the host (K12ΔH, ATCC n° 33767) or on a second compatible plasmid (pACYC derivative). Only in the pUEX vectors is this cI allele present on the vector itself.

It is to be understood that the plasmids presented above ar exemplary and other plasmids or typ s of

xpression vectors maybe employed without departing from the spirit or scope of th present invention.

If a bacteriophage or phagemid is used, instead of plasmid, it should have substantially the same characteristics used to select a plasmid as described above.

EXAMPLE IV : SUBCLONING OF THE P32 ANTIGEN IN PLASMID pIGRI :

Fifteen μg of plasmid "BS-BK-P_{32} complet" (see Example II) was digested with EclXI and BstEII (Boehringer, Mannheim) according to the conditions recommended by the supplier except that at least 3 units of enzyme were used per μg of DNA. EclXI cuts at position 226 (Fig. 5) and BstEII at position 1136, thus approaching very closely the start and stop codon of the mature P_{32} antigen. This DNA is hereafter called DNA coding for the "P_{32} antigen fragment".

The DNA coding for the "P_{32} antigen fragment" (as defined above) is subcloned in pIGRI (see fig. 10a) for expression of a polypeptide devoid of any foreign sequences. To bring the ATG codon of the expression vector in frame with the P_{32} reading frame, an intermediary construct is made in pIG2 (for restriction and genetic map, see fig. 12a; DNA sequences, see fig. 12b; complete restriction site analysis, see Table 7).

Five μg of plasmid pIG2 is digested with NcoI. Its 5' sticky ends are filled in prior to dephosphorylation.

Therefore, the DNA was incubated in 40 μl NB buffer (0.05 M Tris-Cl pH 7.4; 10 mM $MgCl_2$; 0.05% β-mercaptoethanol) containing 0.5 mM of all four dXTP (X = A,T,C,G) and 2 μl of Klenow fragment of *E. coli* DNA polymerase I (5 U/μl, Boehringer, Mannheim) for at least 3 h at 15°C.

After blunting, the DNA was once extract d with one volume of phenol equilibrated against 200 mM Tris-

Cl pH 8, twice with at least two volum s of diethylether and finally collected using the "gene clean kit[T.M.]" (Bio101) as recommended by the supplier. The DNA was then dephosphorylated at the 5' ends in 30 µl of CIP buffer (50 mM TrisCl pH 8, 1 mM $ZnCl_2$) and 20 to 25 units of calf intestine phosphatase (high concentration, Boehringer, Mannheim). The mixture was incubated at 37°C for 30 min, then EGTA (ethyleneglycol bis (β-aminoethylether)-N,N,N',N' tetraacetic acid) pH 8 is added to a final concentration of 10 mM. The mixture was then extracted with phenol followed by diethylether as described above, and the DNA was precipitated by addition of 1/10 volume of 3 M KAc (Ac = CH_3COO) pH 4.8 and 2 volumes of ethanol followed by storage at -20°C for at least one hour.

After centrifugation at 13000 rpm in a Biofuge A (Hereaus) for 5 min the pelleted DNA was dissolved in H_2O to a final concentration of 0.2 µg/µl.

The EclXI-BstEII fragment, coding for the "P_{32} antigen fragment" (see above) was electrophoresed on a 1% agarose gel (BRL) to separate it from the rest of the plasmid and was isolated from the gel by centrifugation over a Millipore HVLP filter (ϕ 2 cm)(2 min,, 13000 rpm, Biofuge at room temperature) and extracted with Tris equilibrated phenol followed by diethylether as described above.

The DNA was subsequently collected using the "Gene clean kit[T.M.]" (Bio101) as recommended by the supplier.

After that, the 5' sticky ends were blunted by treatment with the Klenow fragment of E. coli DNA polymerase I as described above and the DNA was then again collected using the "Gene clean kit[T.M.]" in order to dissolve it in 7 µl of H_2O.

One µl of vector DNA is added together with one µl of 10 x ligase buffer (0.5 M TrisCl pH 7.4, 100 mM $MgCl_2$, 5 mM ATP, 50 mM DTT (dithiothreitol)) and 1 µl

of T4 DNA ligase (1 unit/μl, Boehringer, Mannheim). Ligation was performed for 6 h at 13°C and 5 μl of the mixture is then used to transform strain DH1 (lambda) [strain DH1 - ATCC N° 33849 - lysogenized with wild type bacteriophage λ] using standard transformation techniques as described for instance by Maniatis et al. in "Molecular cloning, a laboratory manual", Cold Spring Harbor Laboratory (1982).

Individual transformants are grown and lysed for plasmid DNA preparation using standard procedures (Experiments with gene fusions, Cold Spring Harbor Laboratory (1984) (T.J. Silhavy, H.L. Berman and L.W. Enquist, eds) and the DNA preparations are checked for the correct orientation of the gene within the plasmid by restriction enzyme analysis.

A check for correct blunting is done by verifying the restoration of the NcoI site at the 5' and 3' end of the antigen coding sequence. One of the clones containing the P_{32} antigen fragment in the correct orientation is kept for further work and designated pIG_2-Mt32. In this intermediary construct, the DNA encoding the antigen is not in frame with the ATG codon. However, it can now be moved as a NcoI fragment to another expression vector.

15 μg of pIG_2-Mt32 is digested with NcoI. The NcoI fragment encoding the P_{32} antigen is gel purified and blunted as described above. After purification, using "gene clear kit TM" it is dissolved in 7 μl of H_2O.

5 μg of plasmid pIGRI is digested with NcoI, blunted and dephosphorylated as described above. After phenol extraction, followed by diethylether and ethanolprecipitation, the pellet is dissolved in H_2O to a final concentration of 0.2 μg/μl.

Ligation of vector and "antigen fragment" DNA is carried out as described above. Th ligation mixture is then transformed into strain DH1 (lambda) and

individual transformants are analysed for the correct orientation of the gene within th plasmid by restriction enzyme analysis. A check for correct blunting is done by verifying the creation of a new <u>NsiI</u> site at the 5' and 3' ends of the antigen coding sequence. One of the clones containing the P_{32} antigen fragment in the correct orientation is kept for further work and designated pIGRI.Mt32.

<u>EXAMPLE V</u>: <u>SUBCLONING OF THE P32 ANTIGEN IN pmTNF MPH:</u>

Fifteen µg of the plasmid pIG2 Mt32 (see example IV) was digested with the restriction enzyme <u>NcoI</u> (Boehringer, Mannheim), according to the conditions recommended by the supplier except that at least 3 units of enzyme were used per µg of DNA.

After digestion, the reaction mixture is extracted with phenol equilibrated against 200mM TrisCl pH 8, (one volume), twice with diethylether (2 volumes) and precipitated by addition of 1/10 volume of 3 M KAc ($Ac=CH_3COO$) pH 4.8 and 2 volumes of ethanol followed by storage at -20°C for at least one hour.

After centrifugation for 5 minutes at 13000 rpm in a Biofuge A (Hereaus) the DNA is electrophoresed on a 1% agarose gel (BRL).

The DNA coding for the "P_{32} antigen fragment" as described above, is isolated by centrifugation over a Millipore HVLP filter (φ 2cm)(2 minutes, 13000 rpm, Biofuge at room temperature) and extracted one with trisCl equilibrated phenol and twice with diethylether. The DNA is subsequently collected using "Gene clean kit T.M." (Bio 101) and dissolved in 7µl of H_2O.

The 5' overhanging ends of the DNA fragment generated by digestion with <u>NcoI</u> were filled in by incubating the DNA in 40 µl NB buffer (0.05 M Tris-HCl, pH 7.4; 10 mM $MgCl_2$; 0.05% β-mercaptoethanol) containing 0.5 mM of all four dXTPS (X = A, T, C, G) and 2µl of Klenow fragment of <u>E. coli</u> DNA polymerase I

(5 units/μl Boehringer Mannheim) for at least 3 h at 15°C. After blunting, the DNA was extracted with phenol, followed by diethylether, and collected using a "gene clean kit T.M." as described above.

Five μg of plasmid pmTNF MPH is digested with StuI, subsequently extracted with phenol, followed by diethylether, and precipitated as described above. The restriction digest is verified by electrophoresis of a 0.5 μg sample on an analytical 1,2% agarose gel.

The plasmid DNA is then desphosphorylated at the 5' ends to prevent self-ligation in 30μl of CIP buffer (50 mM TrisCl pH 8, 1 mM ZnC12) and 20 to 25 units of calf intestine phosphatase (high concentration, Boehringer Mannheim). The mixture is incubated at 37°C for 30 minutes, then EGTA (ethyleneglycol bis (β-aminoethylether)-N,N,N',N' tetraacetic acid) pH8 is added to a final concentration of 10 mM. The mixture is extracted with phenol followed by diethylether and the DNA is precipitated as described above. The precipitate is pelleted by centrifugation in a Biofuge A (Hereaus) at 13000 rpm for 10 min at 4°C and the pellet is dissolved in H_2O to a final DNA concentration of 0.2 μg/μl.

One μl of this vector DNA is mixed with the 7 μl solution containing the DNA fragment coding for the "P32antigen fragment" (as defined above) and 1 μl 10 x ligase buffer (0.5 M TrisCl pH7.4, 100 mM MgC12, 5 mM ATP, 50 mM DTT (dithiothreitol)) plus 1 μl T_4 DNA ligase (1 unit/μl, Boehringer Mannheim) is added. The mixture is incubated at 13°C for 6 hours and 5 μl of the mixture is then used for transformation into strain DH1(lambda) using standard transformation techniques are described by for instance Maniatis et al. in "Molecular cloning, a laboratory manual", Cold Spring Harbor Laboratory (1982).

Individual transformants are grown and then lysed f r plasmid DNA pr paration using standard procedures

(Experiments with gene fusions, Cold Spring Harbor Laboratory 1984 (T.J. Silhavy, M.L. Berman and L.W. Enquist eds.)) and are checked for the correct orientation of the gene within the plasmid by restriction enzyme analysis.

One of the clones containing the DNA sequence encoding the antigen fragment in the correct orientation was retained for further work and designated pmTNF-MPH-Mt32. It encodes all information of the P_{32} antigen starting from position +4 in the amino acid sequence (see fig. 5). The amino acid sequence of the total fusion protein is represented in fig. 13.

<u>EXAMPLE VI</u>: <u>INDUCTION OF ANTIGEN EXPRESSION FROM pmTNF MPH Mt32 :</u>

A- MATERIAL AND METHODS

DNA of pmTNF-MPH-Mt32 is transformed into <u>E. coli</u> strain K12ΔH (ATCC 33767) using standard transformation procedures except that the growth temperature of the cultures is reduced to 28°C and the heat shock temperature to 34°C.

A culture of K12ΔH harboring pmTNF-MPH-Mt32, grown overnight in Luria broth at 28°C with vigorous shaking in the presence of 10 µg/ml tetracycline, is inoculated into fresh Luria broth containing tetracyclin (10 µg/ml) and grown to an optical density at 600 nanometers of 0.2 in the same conditions as for the overnight culture.

When the optical density at 600 nanometers has reached 0.2 half of the culture is shifted to 42°C to induce expression while the other half remains at 28°C as a control. At several time intervals aliquots are taken which are extracted with one volume of phenol equilibrated against M9 salts (0.1% ammonium chloride, 0.3% potassium dihydrogenium phosphate, 1.5% disodium hydrogenium phosphate, 12 molecules of water) and 1% SDS. At th same time, the ptical density (600 nm) f

the culture is checked. The proteins are precipitated from the phen l phase by additi n of two volumes of acetone and storage overnight at -20°C. The precipitate is pelleted (Biofuge A, 5 min., 13000 rpm, room temperature) dried at the air, dissolved in a volume of Laemmli (Nature (1970) 227:680) sample buffer (+ β mercapto ethanol) according to the optical density and boiled for 3 min.

Samples are then run on a SDS polyacrylamide gel (15%) according to Laemmli (1970). Temperature induction of mTNF-His_6-P_{32} is monitored by both Coomassie Brilliant Blue (CBB) staining and immunoblotting. CBB staining is performed by immersing the gel in a 1/10 diluted CBB staining solution (0.5 g CBB-R250 (Serva) in 90 ml methanol : H_2O (1:1 v/v) and 10 ml glacial acetic acid) and left for about one hour on a gently rotating platform. After destaining for a few hours in destaining solution (30% methanol, 7% glacial acetic acid) protein bands are visualised and can be scanned with a densitometer (Ultroscan XL Enhanced Laser Densitometer, LKB).

For immunoblotting the proteins are blotted onto Hybond C membranes (Amersham) as described by Townbin et al (1979). After blotting, proteins on the membrane are temporarily visualised with Ponceau S (Serva) and the position of the molecular weight markers is indicated. The stain is then removed by washing in H_2O. Aspecific protein binding sites are blocked by incubating the blots in 10% non-fat dried milk for about 1 hour on a gently rotating platform. After washing twice with NT buffer (25 mM Tris-HCl, pH 8.0; 150 mM NaCl) blots are incubated with polyclonal rabbit anti-32-kDa antiserum (1:1000), obtained as described in example I ("screening of the λgt11 M. tuberculosis recombinant DNA library with anti-32-kDa antiserum") in the presence of E. coli lysat or with monoclonal

anti-hTNF-antibody which crossreacts with mTNF (Innogenetics, n° 17F5D10) for at least 2 hours on a rotating platform. After washing twice with NT buffer + 0.02% Triton.X.100, blots are incubated for at least 1 hour with the secondary antiserum : alkaline phosphatase-conjugated swine anti-rabbit immunoglobulins (1/500; Prosan) in the first case, and alkaline phosphatase conjugated rabbit anti-mouse immunoglobulins (1/500; Sigma) in the second case.

Blots are washed again twice with NT buffer + 0.02% Triton X100 and visualisation is then performed with nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) from Promega using conditions recommended by the supplier.

B. RESULTS

Upon induction of K12ΔH cells containing pmTNF-MPH-Mt32, a clearly visible band of about 35-kDa appears on CBB stained gels, already one hour after start of induction (Fig. 14a). This band, corresponding to roughly 25% of total protein contents of the cell, reacts strongly with anti-32-kDa and anti-mTNF antisera on immunoblots (Fig. 14b). However, this band represents a cleavage product of the original fusion protein, since a minor band, around 37 kDa, is also visible on immunoblots, reacting specifically with both antisera as well. This suggests that extensive cleavage of the recombinant mTNF-His$_6$-P$_{32}$ takes place about 2-3 kDa from its carboxyterminal end.

<u>EXAMPLE VII</u> : <u>PURIFICATION OF RECOMBINANT ANTIGEN ON IMMOBILIZED METAL ION AFFINITY CHROMATOGRAPHY (IMAC)</u> :

The hybrid protein mTNF-His$_6$-P$_{32}$ (amino acid sequence, see fig. 13) expressed by K12ΔH cells containing pmTNF.MPH.Mt32, is especially designed to facilitate purification by IMAC, since the 6 successive histidines in the polylinker sequence bring about a strong affinity for metal ions (HOCHULI et al, 1988).

a. <u>Preparati n of the crude c ll extract</u> :

12 l of <u>E. coli</u> cells K12ΔH containing plasmid pmTNF-MPH-Mt32 were grown in Luria Broth containing tetracycline (10 μg/ml) at 28°C to an optical density (600 nm) of 0.2 and then induced by shifting the temperature to 42°C. After 3 hours of induction, cells were harvested by centrifugation (Beckman, JA 10 rotor, 7.500 rpm, 10 min). The cell paste was resuspended in lysis buffer (10 mM KCl, 10 mM Tris-HCl pH 6.8, 5 mM EDTA) to a final concentration of 50% (w/v) cells.

ε-NH_2-capronic acid and dithiotreitol (DTT) were added to a final concentration of resp. 20 mM and 1 mM, to prevent proteolytic degradation. This concentrated cell suspension was stored overnight at -70°C.

Cells were lysed by passing them three times through a French press (SLM-Aminco) at a working pressure of 800-1000 psi. During and after lysis, cells were kept systematically on ice.

The cell lysate was cleared by centrifugation (Beckman, JA 20, 18.000 rpm, 20 min, 4°C). The supernatant (SN) was carefully taken off and the pellet, containing membranes and inclusion bodies, was kept for further work since preliminary experiments had shown that the protein was mainly localised in the membrane fraction.

7 M guanidinium hydrochloride (GuHCl, marketed by ICN) in 100 mM phosphate buffer pH 7.2 was added to the pellet volume to a final concentration of 6 M GuHCl. The pellet was resuspended and extracted in a bounce tissue homogenizer (10 cycles).

After clearing (Beckman, JA 20, 18.000 rpm, 20 min, 4°C), about 100 ml of supernatant was collected (= extract 1) and the removing pellet was extracted again as described above (= extract 2, 40 ml).

The different fractions (SN,EX1,EX2) were analysed on SDS-PAGE (Laemmli, Nature 1970; 227:680) together

with control samples of the induced culture. Scanning of the gel revealed that the recombinant protein makes up roughly 25% of the total protein content of the induced cell culture. After fractionation most of the protein was found back in the extracts. No difference was noticed between reducing and non-reducing conditions (plus and minus β-mercaptoethanol).

b. **Preparation of the Ni^{++} IDA (Imino diacetic acid) column** :

5 ml of the chelating gel, Chelating Sepharose 6B (Pharmacia) is washed extensively with water to remove the ethanol in which it is stored and then packed in a "Econo-column" (1 x 10 cm, Biorad). The top of the column is connected with the incoming fluid (sample, buffer, etc) while the end goes to the UV_{280} detector via a peristaltic jump. Fractions are collected using a fraction collector and, when appropriate, pH of the fractions is measured manually.

The column is loaded with Ni^{++} (6 ml $NiCl_2.6H_2O$; 5 $\mu g/\mu l$) and equilibrated with starting buffer (6 M guanidinium hydrochloride, 100 mM phosphate buffer, pH 7.2).

After having applied the sample, the column is washed extensively with starting buffer to remove unbound material.

To elute the bound material, 2 different elution procedures are feasible :

1) elution by decreasing pH,
2) elution by increasing imidazol concentration.

Both will be discussed here.

To regenerate the column, which has to be done after every 2-3 runs, 20 ml (about 5 column volumes) of the following solutions are pumped successively through the column :

- 0.05 M EDTA - 0.5 M NaCl
- 0.1 M NaOH

- H_2O
- 6 ml $NiCl_2.6H_2O$ (5 mg/ml).

After equilibrating with starting buffer the column is ready to use again.

c. <u>Chromatography</u> :

All buffers contained 6 M guanidinium hydrochloride throughout the chromatography. The column was developed at a flow rate of 0.5 ml/min at ambient temperature. Fractions of 2 ml were collected and, when appropriate, further analysed by SDS-PAGE and immunoblotting. Gels were stained with Coomassie Brilliant Blue R250 and silver stain, as described by ANSORGE (1985). Immunoblotting was carried out as described in example I. The primary antiserum used was either polyclonal anti-32kDa-antiserum (1/1000) obtained as described in example I ("screening of the λgt11 <u>M. tuberculosis</u> recombinant DNA library with anti-32kDa-antiserum") or anti-<u>E. coli</u>-immunoglobulins (1/500; PROSAN), or monoclonal anti-hTNF-antibody which cross-reacts with mTNF (Innogenetics, N° 17F5D10). The secondary antiserum was alkaline phosphatase conjugated swine anti-rabbit immunoglobulins (1/500, PROSAN), or alkaline phosphatase conjugated rabbit-anti-mouse immunoglobulins (1/500, Sigma).

C1. Elution with decreasing pH :

Solutions used :

A : 6 M GuHCl 100 mM phosphate pH 7.2

B : 6 M GuHCl 25 mM phosphate pH 7.2

C : 6 M GuHCl 50 mM phosphate pH 4.2

After applying 3 ml of extract 1 (OD_{280} = 32.0) and extensively washing with solution A, the column is equilibrated with solution B and then developed with a linear pH gradient from 7.2 to 4.2 (25 ml of solution B and 25 ml of solution C were mixed in a gradient former). The elution profile is shown in figure 15.

From SDS-PAGE analysis (Coomassie and silverstain) it was clear that most of the originally bound recombinant protein was eluted in the fractions b tween pH 5.3 and 4.7.

Screening of these fractions on immunoblot with anti-32-kDa and the 17F5D10 monoclonal antibody showed that, together with the intact recombinant protein, also some degradation products and higher aggregation forms of the protein were present, although in much lower amount. Blotting with anti-<u>E. coli</u> antibody revealed that these fractions (pH 5.3-4.7) still contained immunodetectable contaminating <u>E. coli</u> proteins (75, 65, 43, 35 and 31 kDa bands) and lipopolysaccharides..

C2. Elution with increasing imidazol concentration :

Solutions used :

A : 6 M GuHCl 100 mM phosphate pH 7.2
B : 6 M GuHCl 50 mM imidazol pH 7.2
C : 6 M GuHCl 100 mM imidazol pH 7.2
D : 6 M GuHCl 15 mM imidazol pH 7.2
E : 6 M GuHCl 25 mM imidazol pH 7.2
F : 6 M GuHCl 35 mM imidazol pH 7.2

Sample application and washing was carried out as in C1, except that after washing, no equilibration was necessary with 6 M GuHCl 25 mM phosphate. The column was first developed with a linear gradient of imidazol going from 0 to 50 mM (25 ml of solution A and 25 ml of solution B were mixed in a gradient former) followed by a step elution to 100 mM imidazol (solution C). During the linear gradient, proteins were gradually eluted in a broad smear, while the step to 100 mM gave rise to a clear peak (fig. 16).

SDS-PAGE analysis of the fractions revealed that in the first part of the linear gradient (fr 1-24) most

contaminating E. coli proteins were washed out, while the latter part of the gradient (fr 25-50) and th 100 mM peak contained more than 90% of the recombinant protein.

As in C1, these fractions showed, besides a major band of intact recombinant protein, some minor bands of degradation and aggregation products. However, in this case, the region below 24-kDa seemed nearly devoid of protein bands, which suggests that less degradation products co-elute with the intact protein. Also, the same contaminating E. coli proteins were detected by immunoblotting, as in C1, although the 31-kDa band seems less intense and even absent in some fractions.

In a second stage, we developed the column with a step gradient of increasing imidazol concentrations. After having applied the sample and washed the column, 2 column volumes (about 8 ml) of the following solutions were brought successively onto the column : solution D, E, F and finally 4 column volumes of solution C. The stepgradient resulted in a more concentrated elution profile (fig. 17) which makes it more suitable for scaling up purposes.

In conclusion, the mTNF-His_6-P_{32} protein has been purified to at least 90% by IMAC. Further purification can be achieved through a combination of the following purification steps :

- IMAC on chelating superose (Pharmacia)
- ion exchange chromatography (anion or cation)
- reversed phase chromatography
- gel filtration chromatography
- immunoaffinity chromatography
- elution from polyacrylamide gel.

These chromatographic methods are commonly used for protein purification.

The plasmids of figures 10b, 11b and 12b are new.

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CLAIMS

1. R combinant polypeptide containing in its polypeptidic chain, one at least of the following amino acid sequences:

- the one extending from the extremity constituted by amino acid at position (-29) to the extremity constituted by amino acid at position (-1) represented on fig. 3a and fig. 3b, or
- the one extending from the extremity constituted by amino acid at position (12) to the extremity constituted by amino acid at position (31) represented on fig. 3a and fig. 3b, or
- the one extending from the extremity constituted by amino acid at position (36) to the extremity constituted by amino acid at position (55) represented on fig. 3a and fig. 3b, or
- the one extending from the extremity constituted by amino acid at position (77) to the extremity constituted by amino acid at position (96) represented on fig. 3a and fig. 3b, or
- the one extending from the extremity constituted by amino acid at position (101) to the extremity constituted by amino acid at position (120) represented on fig. 3a and fig. 3b, or
- the one extending from the extremity constituted by amino acid at position (175) to the extremity constituted by amino acid at position (194) represented on fig. 3a and fig. 3b, or
- the one extending from the extremity constituted by amino acid at position (211) to the extremity constituted by amino acid at position (230) represented on fig. 3a and fig. 3b, or
- the one extending from the extremity constituted by amino acid at position (275) to the extremity c nstitut d by amino acid at position (294) represented on fig. 3a and fig. 3b,

and the peptidic sequences resulting from the modification by substitution and/or by addition and/or by deletion of one or several amino acids in so far as this modification does not alter the following properties :

the polypeptides react with rabbit polyclonal antiserum raised against the protein of 32-kDa of M. bovis BCG culture filtrate, and/or

react selectively with human sera from tuberculosis patients and particularly patients developing an evolutive tuberculosis at an early stage,

and/or react with the amino acid sequence extending from the extremity constituted by amino acid at position (1), to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b.

2. Recombinant polypeptide according to claim 1, containing in its polypeptidic chain, one at least of the following amino acid sequences:

- the one extending from the extremity constituted by amino acid at position (-29) to the extremity constituted by amino acid at position (-1) represented on fig. 4a and fig. 4b, or
- the one extending from the extremity constituted by amino acid at position (12) to the extremity constituted by amino acid at position (31) represented on fig. 4a and fig. 4b, or
- the one extending from the extremity constituted by amino acid at position (36) to the extremity constituted by amino acid at position (55) represented on fig. 4a and fig. 4b, or
- the one extending from the extremity constituted by amino acid at position (77) to the extremity constituted by amino acid at position (96) represented on fig. 4a and fig. 4b, or

- th on xtending from the xtremity constituted by amino acid at position (101) to the extremity constituted by amino acid at position (120) represented on fig. 4a and fig. 4b, or
- the one extending from the extremity constituted by amino acid at position (175) to the extremity constituted by amino acid at position (194) represented on fig. 4a and fig. 4b, or
- the one extending from the extremity constituted by amino acid at position (211) to the extremity constituted by amino acid at position (230) represented on fig. 4a and fig. 4b, or
- the one extending from the extremity constituted by amino acid at position (275) to the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b,

and the peptidic sequences resulting from the modification by substitution and/or by addition and/or by deletion of one or several amino acids in so far as this modification does not alter the following properties :

the polypeptides react with rabbit polyclonal antiserum raised against the protein of 32-kDa of M. bovis BCG culture filtrate, and/or

react selectively with human sera from tuberculosis patients and particularly patients developing an evolutive tuberculosis at an early stage,

and/or react with the amino acid sequence extending from the extremity constituted by amino acid at position (1), to the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b.

3. Recombinant polypeptide according to claim 1, containing in its polypeptidic chain, one at least of th following amino acid s quences:

- the one extending from th extremity constituted by amino acid at position (-30) to th extremity constituted by amino acid at position (-1) represented on fig. 5, or
- the one extending from the extremity constituted by amino acid at position (12) to the extremity constituted by amino acid at position (31) represented on fig. 5, or
- the one extending from the extremity constituted by amino acid at position (36) to the extremity constituted by amino acid at position (55) represented on fig. 5, or
- the one extending from the extremity constituted by amino acid at position (77) to the extremity constituted by amino acid at position (96) represented on fig. 5, or
- the one extending from the extremity constituted by amino acid at position (101) to the extremity constituted by amino acid at position (120) represented on fig. 5, or
- the one extending from the extremity constituted by amino acid at position (175) to the extremity constituted by amino acid at position (194) represented on fig. 5, or
- the one extending from the extremity constituted by amino acid at position (211) to the extremity constituted by amino acid at position (230) represented on fig. 5, or
- the one extending from the extremity constituted by amino acid at position (275) to the extremity constituted by amino acid at position (295) represented on fig. 5,

and the peptidic sequences resulting from the modification by substitution and/or by addition and/or by deleti n of one or several amino acids in so far as

this modification does not alter the following properties :

the polypeptides react with rabbit polyclonal antiserum raised against the protein of 32-kDa of M. bovis BCG culture filtrate, and/or

react selectively with human sera from tuberculosis patients and particularly patients developing an evolutive tuberculosis at an early stage,

and/or react with the amino acid sequence extending from the extremity constituted by amino acid at position (1), to the extremity constituted by amino acid at position (295) represented on fig. 5.

4. Recombinant polypeptide according to claim 1, containing in its polypeptidic chain, one at least of the following amino acid sequences:

- the one extending from the extremity constituted by amino acid at position (1) to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-42) to the extremity constituted by amino acid at position (-1) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-47) to the extremity constituted by amino acid at position (-1) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-49) to to the extremity constituted by amino acid at position (-1) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-55) to the extremity constituted by amino acid at position (-1) represented on fig. 3a and fig. 3b,

- the one extending from th extremity constitut d by amino acid at position (-59) to the extremity constituted by amino acid at position (-1) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-29) to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-42) to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-47) to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-49) to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-55) to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-59) to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b.

5. Recombinant polypeptide according to claim 2, containing in its polypeptidic chain, one at least of the following amino acid sequences:
- the one extending from the extremity constituted by amino acid at position (1) to the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b,

- the one extending from the extremity constituted by amino acid at position (-42) to the extremity constituted by amino acid at position (-1) represented on fig. 4a and fig. 4b,
- the one extending from the extremity constituted by amino acid at position (-47) to the extremity constituted by amino acid at position (-1) represented on fig. 4a and fig. 4b,
- the one extending from the extremity constituted by amino acid at position (-49) to to the extremity constituted by amino acid at position (-1) represented on fig. 4a and fig. 4b,
- the one extending from the extremity constituted by amino acid at position (-55) to the extremity constituted by amino acid at position (-1) represented on fig. 4a and fig. 4b,
- the one extending from the extremity constituted by amino acid at position (-59) to the extremity constituted by amino acid at position (-1) represented on fig. 4a and fig. 4b,
- the one extending from the extremity constituted by amino acid at position (-29) to the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b,
- the one extending from the extremity constituted by amino acid at position (-42) to the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b,
- the one extending from the extremity constituted by amino acid at position (-47) to the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b,
- the one extending from the extremity constituted by amino acid at position (-49) to the extremity c nstituted by amino acid at position (294) represented on fig. 4a and fig. 4b,

- the on extending from the extremity constituted by amino acid at position (-55) to the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b,
- the one extending from the extremity constituted by amino acid at position (-59) to the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b.

6. Recombinant polypeptide according to claim 3, containing in its polypeptidic chain, one at least of the following amino acid sequences:
- the one extending from the extremity constituted by amino acid at position (1) to the extremity constituted by amino acid at position (295) represented on fig. 5,
- the one extending from the extremity constituted by amino acid at position (-43) to the extremity constituted by amino acid at position (-1) represented on fig. 5,
- the one extending from the extremity constituted by amino acid at position (-30) to the extremity constituted by amino acid at position (295) represented on fig. 5,
- the one extending from the extremity constituted by amino acid at position (-43) to the extremity constituted by amino acid at position (295) represented on fig. 5.

7. Recombinant polypeptide according to claim 1, consisting in one of the following amino acid sequences:
- the one extending from the extremity constituted by amino acid at position (-59) to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amin acid at positi n (-59) to the extremity

constituted by amino acid at position (-1) represented on fig. 3a and fig. 3b,

- the one extending from the extremity constituted by amino acid at position (-55) to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-55) to the extremity constituted by amino acid at position (-1) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-49) to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-49) to the extremity constituted by amino acid at position (-1) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-47) to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-47) to the extremity constituted by amino acid at position (-1) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-42) to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b,
- the one extending from the extremity constituted by amino acid at position (-42) to the extremity constituted by amino acid at position (-1) represented on fig. 3a and fig. 3b,
- the one extending fr m th xtremity constituted by amino acid at position (-29) to the extremity

constituted by amino acid at position (294) represented on fig. 3a and fig. 3b,

- the one extending from the extremity constituted by amino acid at position (-29) to the extremity constituted by amino acid at position (-1) represented on fig. 3a and fig. 3b,

- the one extending from the extremity constituted by amino acid at position (1) to the extremity constituted by amino acid at position (294) represented on fig. 3a and fig. 3b,

- the one extending from the extremity constituted by amino acid at position (12) to the extremity constituted by amino acid at position (31) represented on fig. 3a and fig. 3b,

- the one extending from the extremity constituted by amino acid at position (36) to the extremity constituted by amino acid at position (55) represented on fig. 3a and fig. 3b,

- the one extending from the extremity constituted by amino acid at position (77) to the extremity constituted by amino acid at position (96) represented on fig. 3a and fig. 3b,

- the one extending from the extremity constituted by amino acid at position (101) to the extremity constituted by amino acid at position (120) represented on fig. 3a and fig. 3b,

- the one extending from the extremity constituted by amino acid at position (175) to the extremity constituted by amino acid at position (194) represented on fig. 3a and fig. 3b,

- the one extending from the extremity constituted by amino acid at position (211) to the extremity constituted by amino acid at position (230) represented on fig. 3a and fig. 3b,

- the one extending from the extremity constituted by amino acid at position (275) to the extremity

constituted by amino acid at position (294) represented on fig. 3a and fig. 3b.

8. Recombinant polypeptide according to claim 2, consisting in one of the following amino acid sequences:

- the one extending from the extremity constituted by amino acid at position (-59) to the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b,
- the one extending from the extremity constituted by amino acid at position (-59) to the extremity constituted by amino acid at position (-1) represented on fig. 4a and fig. 4b,
- the one extending from the extremity constituted by amino acid at position (-55) to the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b,
- the one extending from the extremity constituted by amino acid at position (-55) to the extremity constituted by amino acid at position (-1) represented on fig. 4a and fig. 4b,
- the one extending from the extremity constituted by amino acid at position (-49) to the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b,
- the one extending from the extremity constituted by amino acid at position (-49) to the extremity constituted by amino acid at position (-1) represented on fig. 4a and fig. 4b,
- the one extending from the extremity constituted by amino acid at position (-47) to the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b,
- the one extending from the extremity constituted by amino acid at position (-47) to th extremity

constituted by amino acid at position (-1) represented on fig. 4a and fig. 4b,

- the one extending from th extremity constituted by amino acid at position (-42) to the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b,

- the one extending from the extremity constituted by amino acid at position (-42) to the extremity constituted by amino acid at position (-1) represented on fig. 4a and fig. 4b,

- the one extending from the extremity constituted by amino acid at position (-29) to the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b,

- the one extending from the extremity constituted by amino acid at position (-29) to the extremity constituted by amino acid at position (-1) represented on fig. 4a and fig. 4b,

- the one extending from the extremity constituted by amino acid at position (1) to the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b,

- the one extending from the extremity constituted by amino acid at position (12) to the extremity constituted by amino acid at position (31) represented on fig. 4a and fig. 4b,

- the one extending from the extremity constituted by amino acid at position (36) to the extremity constituted by amino acid at position (55) represented on fig. 4a and fig. 4b,

- the one extending from the extremity constituted by amino acid at position (77) to the extremity constituted by amino acid at position (96) represented on fig. 4a and fig. 4b,

- the one extending from the extremity constituted by amino acid at position (101) to the extremity

constituted by amino acid at position (120) repr sented on fig. 4a and fig. 4b,

- the one extending from the extremity constituted by amino acid at position (175) to the extremity constituted by amino acid at position (194) represented on fig. 4a and fig. 4b,

- the one extending from the extremity constituted by amino acid at position (211) to the extremity constituted by amino acid at position (230) represented on fig. 4a and fig. 4b,

- the one extending from the extremity constituted by amino acid at position (275) to the extremity constituted by amino acid at position (294) represented on fig. 4a and fig. 4b.

9. Recombinant polypeptide according to claim 3, consisting in one of the following amino acid sequences:

- the one extending from the extremity constituted by amino acid at position (-43) to the extremity constituted by amino acid at position (295) represented on fig. 5,

- the one extending from the extremity constituted by amino acid at position (-43) to the extremity constituted by amino acid at position (-1) represented on fig. 5,

- the one extending from the extremity constituted by amino acid at position (-30) to the extremity constituted by amino acid at position (295) represented on fig. 5,

- the one extending from the extremity constituted by amino acid at position (-30) to the extremity constituted by amino acid at position (-1) represented on fig. 5,

- the one extending from the extremity constituted by amino acid at position (1) to the extremity constituted by amino acid at position (295) represented on fig. 5,

- the one xtending from the extremity constituted by amino acid at position (12) to the extremity constituted by amino acid at position (31) represented on fig. 5,
- the one extending from the extremity constituted by amino acid at position (36) to the extremity constituted by amino acid at position (55) represented on fig. 5,
- the one extending from the extremity constituted by amino acid at position (77) to the extremity constituted by amino acid at position (96) represented on fig. 5,
- the one extending from the extremity constituted by amino acid at position (101) to the extremity constituted by amino acid at position (120) represented on fig. 5,
- the one extending from the extremity constituted by amino acid at position (175) to the extremity constituted by amino acid at position (194) represented on fig. 5,
- the one extending from the extremity constituted by amino acid at position (211) to the extremity constituted by amino acid at position (230) represented on fig. 5,
- the one extending from the extremity constituted by amino acid at position (275) to the extremity constituted by amino acid at position (295) represented on fig. 5.

10. Amino acid sequences constituted by a polypeptide according to claims 1 to 9, and a protein or an heterologous sequence with respect to said polypeptide, said protein or heterologous sequence comprising from about 1 to about 1000 amino acids.

11. Amino acid sequence according to claim 10, wherein the heterologous protein is β-galactosidase.

12. Nucleic acid comprising

- a nucleotide sequence coding for anyone of the polypeptides according to claims 1 to 11,
- or nucleotide sequences which hybridize with the nucleotide sequences coding for anyone of the polypeptides according to claims 1 to 11,
- or nucleotide sequences which are complementary to the nucleotide sequences coding for any of the polypeptides according to claims 1 to 11,
- the above mentioned nucleotide sequences wherein T can be replaced by U.

13. Nucleic acid according to claim 12, comprising one at least of the following nucleotide sequences:
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (182) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (273) to the extremity constituted by nucleotide at position (359) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (360) to the extremity constituted by nucleotide at position (1241) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (1242) to the extremity constituted by nucleotide at position (1358), wherein N represents one of the five A, T, C, G or I nucleotides, represented in fig. 3a and fig. 3b,

or above said nucleotide sequences wherein T is replaced by U,

or nucleic acids which hybridize with said above mentioned nucleotide sequences or the complementary sequences thereof.

14. Nucl ic acid according to claim 13, comprising one at least of the following nucleotide sequences:

- the one extending from the extremity constituted by nucleotide at position (1) to th extremity constituted by nucleotide at position (182) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (273) to the extremity constituted by nucleotide at position (359) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (360) to the extremity constituted by nucleotide at position (1241) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (1242) to the extremity constituted by nucleotide at position (1358), wherein N represents one of the five A, T, C, G or I nucleotides, represented in fig. 4a and fig. 4b,

or above said nucleotide sequences wherein T is replaced by U,

or nucleic acids which hybridize with said above mentioned nucleotide sequences or the complementary sequences thereof.

15. Nucleic acid according to claim 13, comprising one at least of the following nucleotide sequences:

- the one extending from the extremity constituted by nucleotide at position (130) to the extremity constituted by nucleotide at position (219) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (220) to the extremity constituted by nucleotide at position (1104) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (1104) to the extremity constituted by nucleotide at position (1299),

or above said nucleotide sequences wherein T is replaced by U,
or nucleic acids which hybridize with said above mentioned nucleotide sequences or the complementary sequences thereof.

16. Nucleic acid according to claim 13, comprising one of the following sequences:

- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (194) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (195) to the extremity constituted by nucleotide at position (359) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (212) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (213) to the extremity constituted by nucleotide at position (359) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (218) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (219) to the extremity constituted by nucleotide at position (359) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (272) represented in fig. 3a and fig. 3b,

- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (359) r presented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (183) to the extremity constituted by nucleotide at position (359) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (1358) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (1241) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (183) to the extremity constituted by nucleotide at position (1358) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (183) to the extremity constituted by nucleotide at position (1241) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (195) to the extremity constituted by nucleotide at position (1358) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (195) to the extremity constituted by nucleotide at position (1241) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (213) to the extremity constituted by nucleotide at position (1358) represented in fig. 3a and fig. 3b,

- the one extending from the extremity constituted by nucleotid at position (213) to the extremity constituted by nucleotide at position (1241) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (219) to the extremity constituted by nucleotide at position (1358) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (219) to the extremity constituted by nucleotide at position (1241) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (234) to the extremity constituted by nucleotide at position (359) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (234) to the extremity constituted by nucleotide at position (1358) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (234) to the extremity constituted by nucleotide at position (1241) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (273) to the extremity constituted by nucleotide at position (1241) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (273) to the extremity constituted by nucleotide at position (1358) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (360) to the extremity constitut d by nucleotide at position (1358) r pr sent d in fig. 3a and fig. 3b.

17. Nucleic acid according to claim 14, comprising one of the following sequences:

- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (194) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (195) to the extremity constituted by nucleotide at position (359) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (212) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (213) to the extremity constituted by nucleotide at position (359) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (218) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (219) to the extremity constituted by nucleotide at position (359) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (272) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (359) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (183) to the extremity

constituted by nucleotide at position (359) represented in fig. 4a and fig. 4b,

- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (1358) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (1241) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (183) to the extremity constituted by nucleotide at position (1358) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (183) to the extremity constituted by nucleotide at position (1241) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (195) to the extremity constituted by nucleotide at position (1358) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (195) to the extremity constituted by nucleotide at position (1241) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (213) to the extremity constituted by nucleotide at position (1358) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (213) to the extremity constituted by nucleotide at position (1241) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucl otid at position (219) to the extremity

constituted by nucleotid at position (1358) represented in fig. 4a and fig. 4b,

- th on xtending from th extremity constituted by nucleotide at position (219) to the extremity constituted by nucleotide at position (1241) represented in fig. 4a and fig. 4b,

- the one extending from the extremity constituted by nucleotide at position (234) to the extremity constituted by nucleotide at position (359) represented in fig. 4a and fig. 4b,

- the one extending from the extremity constituted by nucleotide at position (234) to the extremity constituted by nucleotide at position (1358) represented in fig. 4a and fig. 4b,

- the one extending from the extremity constituted by nucleotide at position (234) to the extremity constituted by nucleotide at position (1241) represented in fig. 4a and fig. 4b,

- the one extending from the extremity constituted by nucleotide at position (273) to the extremity constituted by nucleotide at position (1241) represented in fig. 4a and fig. 4b,

- the one extending from the extremity constituted by nucleotide at position (273) to the extremity constituted by nucleotide at position (1358) represented in fig. 4a and fig. 4b,

- the one extending from the extremity constituted by nucleotide at position (360) to the extremity constituted by nucleotide at position (1358) represented in fig. 4a and fig. 4b.

18. Nucleic acid according to claim 15, comprising one of the following sequences:

- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (129) represented in fig. 5,

- the one ext nding from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (219) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (1104) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (1299) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (90) to the extremity constituted by nucleotide at position (219) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (90) to the extremity constituted by nucleotide at position (1299) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (90) to the extremity constituted by nucleotide at position (1104) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (130) to the extremity constituted by nucleotide at position (1104) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (130) to the extremity constituted by nucleotide at position (1299) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (220) to the extremity constituted by nucleotide at position (1299) represented in fig. 5.

19. Nucleic acid according to claim 13, consisting in one of the following nucl otide sequences:

- the on xtending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (182) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (194) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (212) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (218) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (272) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (359) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (1241) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (1358) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (183) to the extremity constituted by nucleotide at position (359) represented in fig. 3a and fig. 3b,

- th one extending from the extremity constituted by nucleotide at position (183) to the extremity constituted by nucleotide at position (1241) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (183) to the extremity constituted by nucleotide at position (1358) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (195) to the extremity constituted by nucleotide at position (359) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (195) to the extremity constituted by nucleotide at position (1241) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (195) to the extremity constituted by nucleotide at position (1358) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (213) to the extremity constituted by nucleotide at position (359) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (213) to the extremity constituted by nucleotide at position (1241) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (213) to the extremity constituted by nucleotide at position (1358) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (219) to the extremity constituted by nucleotide at position (359) repr sented in fig. 3a and fig. 3b,

- the one extending from the extremity constituted by nucleotid at position (219) to the extremity constituted by nucleotide at position (1241) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (219) to the extremity constituted by nucleotide at position (1358) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (234) to the extremity constituted by nucleotide at position (359) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (234) to the extremity constituted by nucleotide at position (1241) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (234) to the extremity constituted by nucleotide at position (1358) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (273) to the extremity constituted by nucleotide at position (359) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (273) to the extremity constituted by nucleotide at position (1241) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (273) to the extremity constituted by nucleotide at position (1358) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (360) to the extremity constituted by nucleotide at position (1241) represented in fig. 3a and fig. 3b,

- the ne extending from the extremity constituted by nucleotide at position (360) to th extremity constituted by nucleotide at position (1358) represented in fig. 3a and fig. 3b,
- the one extending from the extremity constituted by nucleotide at position (1242) to the extremity constituted by nucleotide at position (1358) represented in fig. 3a and fig. 3b.

20. Nucleic acid according to claim 14, consisting in one of the following nucleotide sequences:
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (182) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (194) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (212) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (218) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (272) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (359) represented in fig. 4a and fig. 4b,
- the on xt nding from th extremity constituted by nucl otid at p sition (1) to the extremity constituted

by nucleotide at position (1241) represented in fig. 4a and fig. 4b,

- the on ext nding from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (1358) represented in fig. 4a and fig. 4b,

- the one extending from the extremity constituted by nucleotide at position (183) to the extremity constituted by nucleotide at position (359) represented in fig. 4a and fig. 4b,

- the one extending from the extremity constituted by nucleotide at position (183) to the extremity constituted by nucleotide at position (1241) represented in fig. 4a and fig. 4b,

- the one extending from the extremity constituted by nucleotide at position (183) to the extremity constituted by nucleotide at position (1358) represented in fig. 4a and fig. 4b,

- the one extending from the extremity constituted by nucleotide at position (195) to the extremity constituted by nucleotide at position (359) represented in fig. 4a and fig. 4b,

- the one extending from the extremity constituted by nucleotide at position (195) to the extremity constituted by nucleotide at position (1241) represented in fig. 4a and fig. 4b,

- the one extending from the extremity constituted by nucleotide at position (195) to the extremity constituted by nucleotide at position (1358) represented in fig. 4a and fig. 4b,

- the one extending from the extremity constituted by nucleotide at position (213) to the extremity constituted by nucleotide at position (359) represented in fig. 4a and fig. 4b,

- the one extending from the extremity constituted by nucleotide at position (213) to the extremity

constituted by nucleotide at position (1241) represented in fig. 4a and fig. 4b,

- the one extending from the extremity constituted by nucleotide at position (213) to the extremity constituted by nucleotide at position (1358) represented in fig. 4a and fig. 4b,

- the one extending from the extremity constituted by nucleotide at position (219) to the extremity constituted by nucleotide at position (359) represented in fig. 4a and fig. 4b,

- the one extending from the extremity constituted by nucleotide at position (219) to the extremity constituted by nucleotide at position (1241) represented in fig. 4a and fig. 4b,

- the one extending from the extremity constituted by nucleotide at position (219) to the extremity constituted by nucleotide at position (1358) represented in fig. 4a and fig. 4b,

- the one extending from the extremity constituted by nucleotide at position (234) to the extremity constituted by nucleotide at position (359) represented in fig. 4a and fig. 4b,

- the one extending from the extremity constituted by nucleotide at position (234) to the extremity constituted by nucleotide at position (1241) represented in fig. 4a and fig. 4b,

- the one extending from the extremity constituted by nucleotide at position (234) to the extremity constituted by nucleotide at position (1358) represented in fig. 4a and fig. 4b,

- the one extending from the extremity constituted by nucleotide at position (273) to the extremity constituted by nucleotide at position (359) represented in fig. 4a and fig. 4b,

- th one extending from the extremity constituted by nucl otide at position (273) to th xtremity

constituted by nucleotide at position (1241) represented in fig. 4a and fig. 4b,
- the on extending from the extremity constituted by nucleotide at position (273) to the extremity constituted by nucleotide at position (1358) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (360) to the extremity constituted by nucleotide at position (1241) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (360) to the extremity constituted by nucleotide at position (1358) represented in fig. 4a and fig. 4b,
- the one extending from the extremity constituted by nucleotide at position (1242) to the extremity constituted by nucleotide at position (1358) represented in fig. 4a and fig. 4b.

21. Nucleic acid according to claim 15, consisting in one of the following nucleotide sequences:
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (129) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (219) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (1104) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (1299) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (90) to the extremity constituted by nucleotide at position (219) represented in fig. 5,

- the one extending from the extremity constituted by nucleotide at position (90) to the extremity constituted by nucleotide at position (1104) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (90) to the extremity constituted by nucleotide at position (1299) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (130) to the extremity constituted by nucleotide at position (219) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (130) to the extremity constituted by nucleotide at position (1104) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (130) to the extremity constituted by nucleotide at position (1299) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (220) to the extremity constituted by nucleotide at position (1104) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (220) to the extremity constituted by nucleotide at position (1299) represented in fig. 5,
- the one extending from the extremity constituted by nucleotide at position (1104) to the extremity constituted by nucleotide at position (1299) represented in fig. 5.

22. Recombinant nucleic acid containing at least one of the nucleotide sequences according to claims 13 to 21, inserted in a heterologous nucleic acid.

23. DNA or RNA primer constituted by one of the following sequences:

A(i) CAGCTTGTTGACAGGGTTCGTGGC
A(ii) GGTTCGTGGCGCCGTCACG
A(iii) CGTCGCGCGCCTAGTGTCGG
A(iv) CGGCGCCGGTCGGTGGCACGGCGA
A(v) CGTCGGCGCGGCCCTAGTGTCGG

B TCGCCCGCCCTGTACCTG

C GCGCTGACGCTGGCGATCTATC

D CCGCTGTTGAACGTCGGGAAG

E AAGCCGTCGGATCTGGGTGGCAAC

F(i) ACGGCACTGGGTGCCACGCCCAAC
F(ii) ACGCCCAACACCGGGCCCGCCGCA
F(iii) ACGGGCACTGGGTGCCACGCCCAAC
F(iv) ACGCCCCAACACCGGGCCCGCGCCCCA

24. DNA or RNA primer set constituted by any of the nucleotide sequences A(i), A(ii), A(iii), A(iv), A(v), B, C, D, E, F(i), F(ii), F(iii) or F(iv) in association with the complement of any other nucleotide sequences chosen from A, B, C, D, E, or F, A meaning any of the sequences A(i), A(ii), A(iii), A(iv), A(v) and F any of the sequences F(i), F(ii), F(iii) and F(iv),

A(i), A(ii), A(iii), A(iv), A(v), B, C, D, E, F(i), F(ii), F(iii) and F(iv) having the meaning of claim 11, and

advantageously constituted by the following elements :

A(i)
or A(ii)
or A(iii) and the complement of B
or A(iv)
or A(v)

A(i)
or A(ii)
or A(iii) and the complement of C
or A(iv)
or A(v)

B and the complement of C

A(i)
or A(ii)
or A(iii) and the complement of F
or A(iv)
or A(v)

A(i)
or A(ii)
or A(iii) and the complement of D
or A(iv)
or A(v)

A(i)
or A(ii)
or A(iii) and the complement of E
or A(iv)
or A(v)

B and the complement of D
B and the complement of E
B and the complement of F
C and the complement of D
C and the complement of E
C and the complement of F
D and the complement of E
D and the complement of F
E and the complement of F.

25. Recombinant vector, particularly for cloning and/or expression, comprising a vector sequence,

notably of the type plasmid, cosmid or phage, and a recombinant nucleic acid according to anyone of claims 13 to 21, in one of the non ssential sit s for its replication.

26. Recombinant vector according to claim 25, containing in one of its non essential sites for its replication necessary elements to promote the expression of polypeptides according to anyone of claims 1 to 12 in a cellular host and possibly a promoter recognized by the polymerase of the cellular host, particularly an inductible promoter and possibly a signal sequence and/or an anchoring sequence.

27. Recombinant vector according to claim 26, containing the elements enabling the expression by <u>E. coli</u> of a nucleic acid according to anyone of claims 6 to 9 inserted in the vector, and particularly the elements enabling the expression of the gene or part thereof of β-galactosidase.

28. Cellular host which is transformed by a recombinant vector according to anyone of claims 25 to 27, and comprising the regulation elements enabling the expression of the nucleotide sequence coding for the polypeptide according to anyone of claims 1 to 12 in this host.

29. Cellular host according to claim 28, chosen from among bacteria such as <u>E. coli</u>, transformed by the vector according to claim 25, or chosen from among eukaryotic organism, transformed by the vector according to claim 25.

30. Expression product of a nucleic acid expressed by a transformed cellular host according to anyone of claims 28 or 29.

31. Antibody characterized by the fact that it is directed against a recombinant polypeptide according to anyone of claims 1 to 12.

32. Nucle tidic prob s, hybridizing with anyone of th nucleic acids according to claims 13 to 21 or with their complementary sequences,
and particularly the probes chosen among the following nucleotidic sequences

Probes A(i), A(ii), A(iii) and A(iv)

A(i) CAGCTTGTTGACAGGGTTCGTGGC
A(ii) GGTTCGTGGCGCCGTCACG
A(iii) CGTCGCGCGCCTAGTGTCGG
A(iv) CGGCGCCGTCGGTGGCACGGCGA
A(v) CGTCGGCGCGGCCCTAGTGTCGG

Probe B

TCGCCCGCCCTGTACCTG

Probe C

GCGCTGACGCTGGCGATCTATC

Probe D

CCGCTGTTGAACGTCGGGAAG

Probe E

AAGCCGTCGGATCTGGGTGGCAAC

Probes F(i) and F(ii)

F(i) ACGGCACTGGGTGCCACGCCCAAC
F(ii) ACGCCCAACACCGGGCCCGCCGCA
F(iii) ACGGGCACTGGGTGCCACGCCCAAC
F(iv) ACGCCCCAACACCGGGCCCGCGCCCCA
or their complementary nucleotidic sequences.

33. Process for preparing a recombinant polypeptide according to anyone of claims 1 to 12 comprising the following steps:

- the culture in an appropriate medium of a cellular host which has previously been transformed by an appropriate vector containing a nucleic acid according to anyone of claims 12 to 22, and
- the recovery of the polypeptide produced by the above said transformed cellular host from the above said culture medium.

34. Method for the _in vitro_ diagnostic of tuberculosis in a patient liable to be infected by Mycobacterium tuberculosis comprising the following steps:

- the possible previous amplification of the amount of the nucleotide sequences according to anyone of claims 12 to 22, liable to be contained in a biological sample taken from said patient by means of a DNA primer set according to claim 24,
- contacting the above mentioned biological sample with a nucleotide probe according to claim 32, under conditions enabling the production of an hybridization complex formed between said probe and said nucleotide sequence,
- detecting the above said hybridization complex which has been possibly formed.

35. Method for the _in vitro_ diagnostic of tuberculosis in a patient liable to be infected by Mycobacterium tuberculosis comprising

- contacting a biological sample taken from a patient with a polypeptide according to anyone of claims 1 to 11, under conditions enabling an _in vitro_ immunological reaction between said polypeptide and the antibodies which are possibly present in the biological sample and
- the _in vitro_ detection of the antigen/antibody complex which has been possibly formed.

36. Method for the _in vitro_ diagnostic of tuberculosis in a patient liable to be infected by _M. tuberculosis_, comprising th following steps :

- contacting the biological sample with an appropriate antibody according to claim 31, under conditions enabling an in vitro immunological reaction between said antibody and the antigens of M. tuberculosis which are possibly present in the biological sample and
- the in vitro detection of the antigen/antibody complex which may be formed.

37. Necessary or kit for an in vitro diagnostic method of tuberculosis in a patient liable to be infected by Mycobacterium tuberculosis according to claim 34, comprising
- a determined amount of a nucleotide probe according to claim 32,
- advantageously the appropriate medium for creating an hybridization reaction between the sequence to be detected and the above mentioned probe,
- advantageously, reagents enabling the detection of the hybridization complex which has been formed between the nucleotide sequence and the probe during the hybridization reaction.

38. Necessary or kit for an in vitro diagnostic method of tuberculosis in a patient liable to be infected by Mycobacterium tuberculosis according to claim 35, comprising
- a polypeptide according to anyone of claims 1 to 11,
- reagents for making a medium appropriate for the immunological reaction to occur,
- reagents enabling to detect the antigen/antibody complex which has been produced by the immunological reaction, said reagents possibly having a label, or being liable to be recognized by a labeled reagent, more particularly in the case where the above mentioned polypeptide is not labeled.

39. Necessary or kit for an in vitro diagnostic method of tuberculosis in a patient liabl to be

infected by Mycobacterium tuberculosis according to claim 36, comprising

- an antibody according to claim 31,
- reagents for making a medium appropriate for the immunological reaction to occur,
- reagents enabling to detect the antigen/antibody complexes which have been produced by the immunological reaction, said reagents possibly having a label or being liable to be recognized by a label reagent, more particularly in the case where the above mentioned antibody is not labeled.

40. Immunogenic composition comprising a polypeptide according to anyone of claims 1 to 11, in association with a pharmaceutically acceptable vehicle.

41. Vaccine composition comprising among other immunogenic principles anyone of the polypeptides according to claims 1 to 11 or the expression product of claim 30, possibly coupled to a natural protein or to a synthetic polypeptide having a sufficient molecular weight so that the conjugate is able to induce _in vivo_ the production of antibodies neutralizing Mycobacterium tuberculosis, or induce _in vivo_ a cellular immune response by activating M. tuberculosis antigen-responsive T cells.

42. Process for the enzymatical amplification of a nucleotide sequence according to claims 12 to 22, and detection of the amplified nucleotide sequence, wherein

- the amplification is achieved by PCR technique by means of a primer set and the detection of the PCR amplified product is achieved by a hybridization reaction with a detection probe constituted by an oligonucleotide sequence of at least 10 nucleotides, said sequence being located between the two PCR primers which have been used for amplifying said nucleotide sequence,

- the primer set and detection probe used being preferably chosen among th following lements:

<u>Primer set</u>

P1 GAGTACCTGCAGGTGCCGTCGCCGTCGATGGGCCG

P2 compl. GTACCACTCGAACGCCGGGGTGTTGAT

<u>Probe B</u>

TCGCCCGCCCTGTACCTG

<u>Primer set</u>

P1 GAGTACCTGCAGGTGCCGTCGCCGTCGATGGGCCG

P3 compl. TCCCACTTGTAAGTCTGGCA

<u>Probe B</u>

TCGCCCGCCCTGTACCTG

<u>Primer set</u>

P1 GAGTACCTGCAGGTGCCGTCGCCGTCGATGGGCCG

P4 compl. CGGCAGCTCGCTGGTCAGGA

<u>Probe B</u>

TCGCCCGCCCTGTACCTG

<u>Primer set</u>

P1 GAGTACCTGCAGGTGCCGTCGCCGTCGATGGGCCG

P5 compl. GCGTCACCCATCGCCAGGCCGATCAGG

<u>Probe B</u>

TCGCCCGCCCTGTACCTG or

<u>Probe C</u>

GCGCTGACGCTGGCGATCTATC

<u>Primer set</u>

P1 GAGTACCTGCAGGTGCCGTCGCCGTCGATGGGCCG

P6 compl. GCGCCCCAGTACTCCCAGCTGTGCGT

<u>Probe B</u>

TCGCCCGCCCTGTACCTG or

<u>Probe C</u>

GCGCTGACGCTGGCGATCTATC or

<u>Probe D</u>

CCGCTGTTGAACGTCGGGAAG or

Probe E

AAGCCGTCGGATCTGGGTGGCAAC

Primer set

P2 ATCAACACCCCGGCGTTCGAGTGGTAC

P5 compl. GCGTCACCCATCGCCAGGCCGATCAGG

Probe C

GCGCTGACGCTGGCGATCTATC

Primer set

P2 ATCAACACCCCGGCGTTCGAGTGGTAC

P6 compl. GCGCCCCAGTACTCCCAGCTGTGCGT

Probe C

GCGCTGACGCTGGCGATCTATC or

Probe D

CCGCTGTTGAACGTCGGGAAG or

Probe E

AAGCCGTCGGATCTGGGTGGCAAC

Primer set

P3 TGCCAGACTTACAAGTGGGA

P5 compl. GCGTCACCCATCGCCAGGCCGATCAGG

Probe C

GCGCTGACGCTGGCGATCTATC

Primer set

P3 TGCCAGACTTACAAGTGGGA

P6 compl. GCGCCCCAGTACTCCCAGCTGTGCGT

Probe C

GCGCTGACGCTGGCGATCTATC or

Probe D

CCGCTGTTGAACGTCGGGAAG or

Probe E

AAGCCGTCGGATCTGGGTGGCAAC

Primer set

P4 TCCTGACCAGCGAGCTGCCG

P5 compl. GCGTCACCCATCGCCAGGCCGATCAGG

Probe C

GCGCTGACGCTGGCGATCTATC

Primer set

P4 TCCTGACCAGCGAGCTGCCG

P6 compl. GCGCCCCAGTACTCCCAGCTGTGCGT

Probe C

GCGCTGACGCTGGCGATCTATC or

Probe D

CCGCTGTTGAACGTCGGGAAG or

Probe E

AAGCCGTCGGATCTGGGTGGCAAC

Primer set

P5 CCTGATCGGCCTGGCGATGGGTGACGC

P6 compl. GCGCCCCAGTACTCCCAGCTGTGCGT

Probe D

CCGCTGTTGAACGTCGGGAAG or

Probe E

AAGCCGTCGGATCTGGGTGGCAAC

or the primer set being preferably chosen among the primer sets according to claim 24, and the detection probe being constituted by any oligonucleotide sequence of at least 10 nucleotides, said sequence being located between the two PCR primers constituting the primer set which has been used for amplifying said nucleotide sequence.

43. A vector sequence forming part of a recombinant vector according to claim 25, said vector sequence having either the nucleic acid sequence represented in fig. 10b, or the nucleic acid sequence represented in fig. 11b, or the nucleic acid sequence represented in fig. 12b.

44. Plasmids comprising either the nucleic acid sequence of fig. 10b, or the nucleic acid sequence of fig. 11b, or the nucleic acid s quence of fig. 12b.

45. Peptides of claim 1, advantageously used to produce antibodies, particularly monoclonal antibodies and which have the following amino acid sequences :

Amino acid position (NH_2-terminal)		Amino acid position (COOH-terminal)
12	QVPSPSMGRDIKVQFQSGGA	31
36	LYLLDGLRAQDDFSGWDINT	55
77	SFYSDWYQPACRKAGCQTYK	96
101	LTSELPGWLQANRHVKPTGS	120
175	KASDMWGPKEDPAWQRNDPL	194
211	CGNGKPSDLGGNNLPAKFLE	230
275	KPDLQRHWVPRPTPGPPQGA	294
77	SFYSDWYQPACGKAGCQTYK	96
276	PDLQRALGATPNTGPAPQGA	299

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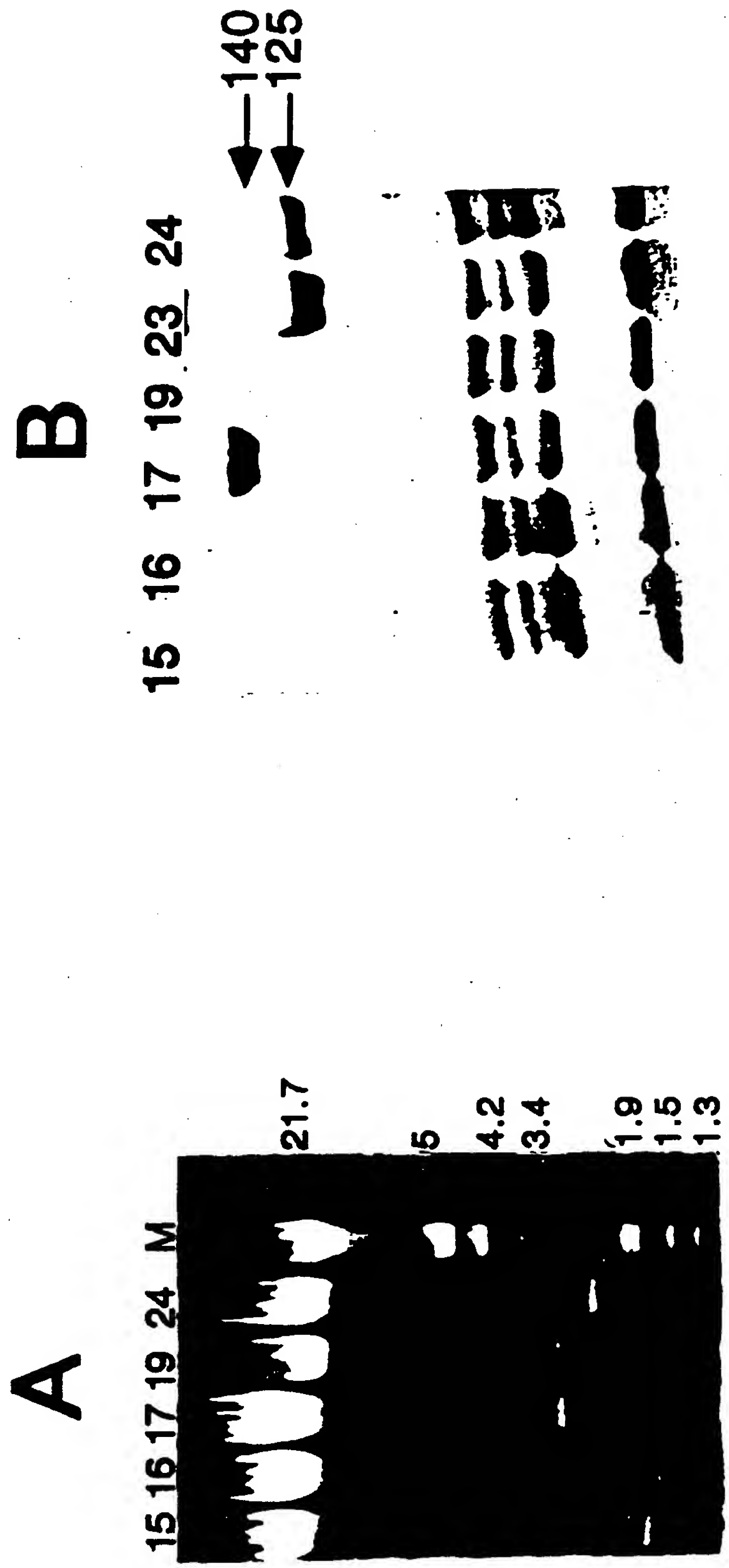
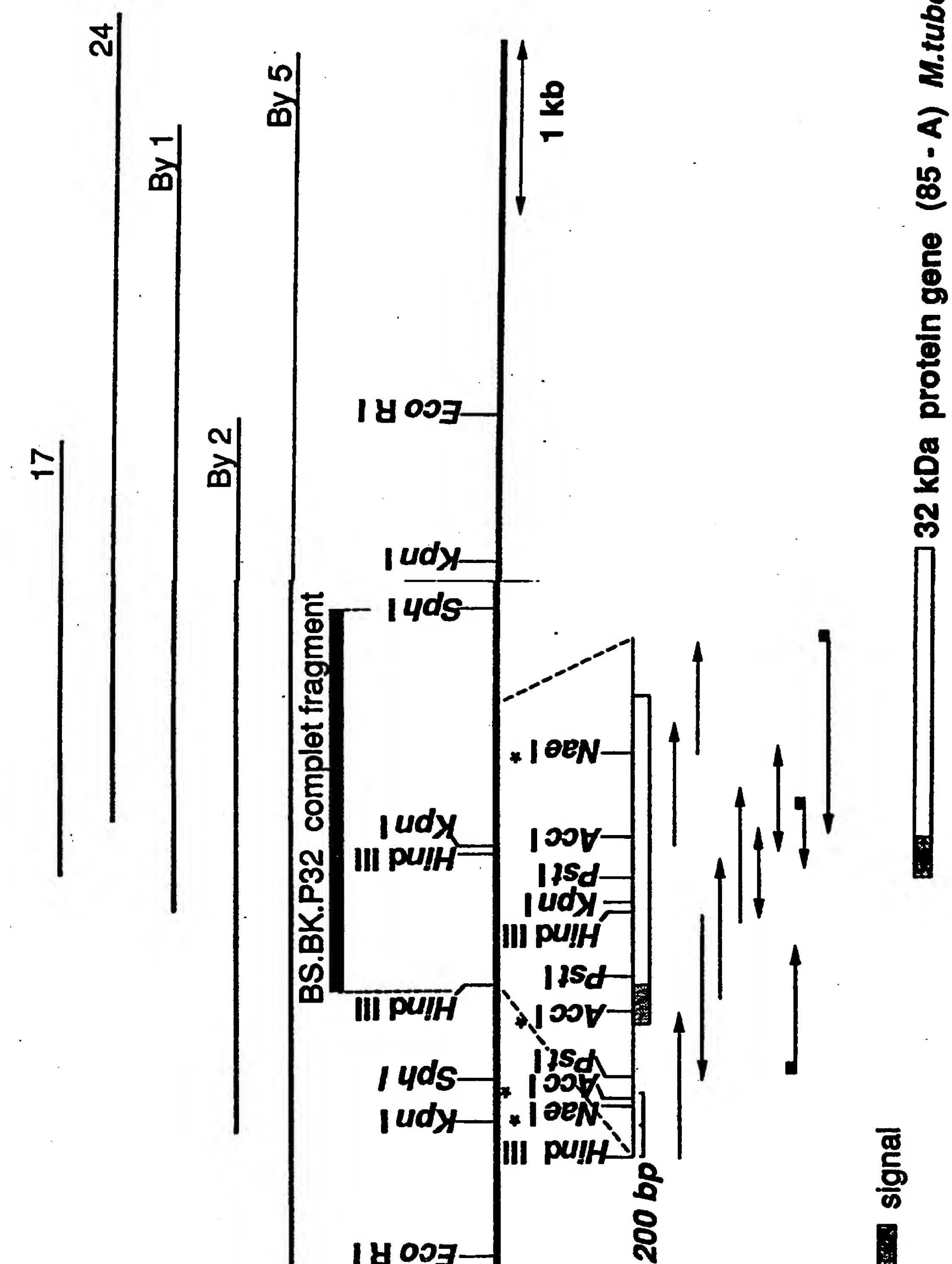


Figure 1

Figure 2


32 kDa protein gene (85 - A) M.tuberculosis
1 kb
24
By 5
By 1
By 2
17
Eco R I
Kpn I
Sph I
BS.BK.P32 complet fragment
Nae I *
Kpn I
Hind III
Acc I
Pst I
Kpn I
Hind III
Pst I
Hind III
Acc I *
Sph I
Pst I
Acc I
Kpn I
Nae I *
Hind III
200 bp
Eco R I
signal

CGACACATGCCCAGACACTGCGGAAATGCCACCTTCAGGCCGTCGCGTCGGT
CCCGAA $\boxed{TTGGC}$ CGTGAACGACCGCCGG $\boxed{ATAA}$ GGGTTTCGGCGGTGCGCTTGATGCGGGT

GGACGCCCAAGTTGTGGTTGACTACACGAGCACTGCCGGGCCCAGCGCCTGCAGTCTGACCT
183 195 213 219
AATTCAGGATGCGCCCAAACATGCATGGATGCG $\boxed{TTGAGA}$ TGAGGATGAGGGAGCA $\boxed{AGA}$
MET-ARG-PRO—ASN-MET-HIS- GLY-CYS-VAL— GLU- MET- ARG-MET—ARG—GLU-ALA—ARG
-59 -55 -49 -47

234 $\boxed{AT}$G-CAG-CTT-GTT-GAC-AGG-GTT-CGT-GGC-GCC-GTC-ACG-GGT-ATG-TCG-CGT-CGA-CTC-GTG-GTC-
-42 *MET-GLN-LEU-VAL-ASP-ARG-VAL-ARG-GLY-ALA-VAL-THR-GLY-MET-SER-ARG-ARG-LEU-VAL-VAL-*
-29

294 GGG-GCC-GTC-XCG-CXC-YTA-GTG-TCG-GGT-CTG-GTC-GGC-GCC-GTC-GGT-GGC-ACG-GCG-ACC-GCG-
-22 *GLY-ALA-VAL-* a_1 - b_1 *-LEU-VAL-SER-GLY-LEU-VAL-GLY-ALA-VAL-GLY-GLY-THR-ALA-THR-ALA-*

354 GGG-GCA-TTT-TCC-CGG-CCG-GGC-TTG-CCG-GTG-GAG-TAC-CTG-CAG-GTG-CCG-TCG-CCG-TCG-ATG-
-2 *GLY-ALA-*phe-ser-arg-pro-gly-leu-pro-val-glu-tyr-leu-gln-val-pro-ser-pro-ser-met-
-1 +1

414 GGC-CGT-GAC-ATC-AAG}-GTC-CAA-TTC-CAA-AGT-GGT-GGT-GCC-AAC-TCG-CCC-GCC-CTG-TAC-CTG-
19 gly-arg-asp-ile-lys -val-gln-phe-gln-ser-gly-gly-ala-asn-ser-pro-ala-leu-tyr-l u-
↓ 17

474 CTC-GAC-GGC-CTG-CGC-GCG-CAG-GAC-GAC-TTC-AGC-GGC-TGG-GAC-ATC-AAC-ACC-CCG-GCG-TTC-
39 leu-asp-gly-leu-arg-ala-gln-asp-asp-phe-ser-gly-trp-asp-ile-asn-thr-pro-ala-ph -

534 GAG-TGG-TAC-GAC-CAG-TCG-GGC-CTG-TCG-GTG-GTC-ATG-CCG-GTG-GGT-GGC-CAG-TCA-AGC-TTC-
59 glu-trp-tyr-asp-gln-ser-gly-leu-ser-val-val-met-pro-val-gly-gly-gln-ser-ser-ph -

594 TAC-TCC-GAC-TGG-TAC-CAG-CCC-GCC-TGC-ZGC-AAG-GCC-GGT-TGC-CAG-{ACT-TAC-AAG-TGG-GAG-
79 tyr-ser-asp-trp-tyr-gln-pro-ala-cys- a_2 -lys-ala-gly-cys-gln- thr-tyr-lys-trp-glu-

Figure 3a

654 ACC-TTC-CTG-ACC-AGC-GAG-CTG-CCG-GGG-TGG-CTG-CAG-GCC-AAC-AGG-CAC-GTC-AAG-CCC-ACC-
99 thr-phe-leu-thr-ser-glu-leu-pro-gly-trp-leu-gln-ala-asn-arg-his-val-lys-pro-thr-

714 GGA-AGC-GCC-GTC-GTC-GGT-CTT-TCG-ATG-GCT-GCT-TCT-TCG-GCG-CTG-ACG-CTG-GCG-ATC-TAT-
119 gly-ser-ala-val-val-gly-leu-ser-met-ala-ala-ser-ser-ala-leu-thr-leu-ala-il -tyr-

774 CAC-CCC-CAG-CAG-TTC-GTC-TAC-GCG-GGA-GCG-ATG-TCG-GGC-CTG-TTG-GAC-CCC-TCC-CAG-GCG-
139 his-pro-gln-gln-phe-val-tyr-ala-gly-ala-met-ser-gly-leu-leu-asp-pro-ser-gln-ala-

834 ATG-GGT-CCC-ACC-CTG-ATC-GGC-CTG-GCG-ATG-GGT-GAC-GCT-GGC-GGC-TAC-AAG-GCC-TCC-GAC-
159 met-gly-pro-thr-leu-ile-gly-leu-ala-met-gly-asp-ala-gly-gly-tyr-lys-ala-ser-asp-

894 ATG-TGG-GGC-CCG-AAG-GAG-GAC-CCG-GCG-TGG-CAG-CGC-AAC-GAC-CCG-CTG-TTG-AAC-GTC-GGG-
179 met-trp-gly-pro-lys-glu-asp-pro-ala-trp-gln-arg-asn-asp-pro-leu-leu-asn-val-gly-

954 AAG-CTG-ATC-GCC-AAC-AAC-ACC-CGC-GTC-TGG-GTG-TAC-TGC-GGC-AAC-GGC-AAG-CCG-TCG-GAT-
199 lys-leu-ile-ala-asn-asn-thr-arg-val-trp-val-tyr-cys-gly-asn-gly-lys-pro-ser-asp-

↓ 24

1014 CTG-GGT-GGC-AAC-AAC-CTG-CCG-GCC-AAG-TTC-CTC-GAG-GGC-TTC-GTG-CGG-ACC-AGC-AAC-ATC-
219 leu-gly-gly-asn-asn-leu-pro-ala-lys-phe-leu-glu-gly-phe-val-arg-thr-ser-asn-il -

1074 AAG-TTC-CAA-GAC-GCC-TAC-AAC-GCC-GGT- GGW-ZGC -CAC-AAC-GGC-GTG-TTC-GAC-TTC-CCG-GAC-
239 lys-phe-gln-asp-ala-tyr-asn-ala-gly-gly- a_2 -his-asn-gly-val-phe-asp-phe-pro-asp-

1134 AGC-GGT-ACG-CAC-AGC-TGG-GAG-TAC-TGG-GGC-GCG-CAG-CTC-AAC-GCT-ATG-AAG-CCC-GAC-CTG-
259 ser-gly-thr-his-ser-trp-glu-tyr-trp-gly-ala-gln-leu-asn-ala-met-lys-pro-asp-leu-

1242

1194 CAA-CG -CAC-TGG-GTG-CCA-CGC-CCA-ACA-CCG-GGC-CCG- KCL -CAG-GGC-GCC-TAGCTCCGAACAGACA
279 gln-arg- a_3 - b_3 - c_3 - d_3 - e_3 - f_3 -thr- a_4 -gly-pro- a_5 -gln-gly-ala-TER

294

1258 CAACATCTAGCNNCGGTGACCCTTGTGGNNCANATGTTTCCTAAATCCCGTCCCTAGCTCCCGCNGCNNCCGTGTGGTTA
1338 GCTACCTGACNNCATGGGTTT 1358

Figure 3b

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               CGACACATGCCCAGACACTGCGGAAATGCCACCTTCAGGCCGTCGCGTCGGT
     CCCGAATTGGCCGTGAACGACCGCCGGATAAGGGTTTCGGCGGTGCGCTTGATGCGGGT

     GGACGCCCAAGTTGTGGTTGACTACACGAGCACTGCCGGGCCCAGCGCCTGCAGTCTGACCT
               183         195                  213    219
     AATTCAGGATGCGCCCAAACATGCATGGATGCGTTGAGATGAGGATGAGGGAGCAAGA
             MET-ARG-PRO—ASN-MET-HIS- GLY-CYS-VAL— GLU- MET- ARG-MET—ARG—GLU-ALA—ARG
              -59         -55                          -49     -47

234  ATG-CAG-CTT-GTT-GAC-AGG-GTT-CGT-GGC-GCC-GTC-ACG-GGT-ATG-TCG-CGT-CGA-CTC-GTG-GTC-
-42  MET-GLN-LEU-VAL-ASP-ARG-VAL-ARG-GLY-ALA-VAL-THR-GLY-MET-SER-ARG-ARG-LEU-VAL-VAL-
                                                         -29

294  GGG-GCC-GTC-GCG-CGC-CTA-GTG-TCG-GGT-CTG-GTC-GGC-GCC-GTC-GGT-GGC-ACG-GCG-ACC-GCG-
-22  GLY-ALA-VAL-ALA-ARG-LEU-VAL-SER-GLY-LEU-VAL-GLY-ALA-VAL-GLY-GLY-THR-ALA-THR-ALA-

354  GGG-GCA-TTT-TCC-CGG-CCG-GGC-TTG-CCG-GTG-GAG-TAC-CTG-CAG-GTG-CCG-TCG-CCG-TCG-ATG-
 -2  GLY-ALA-phe-ser-arg-pro-gly-leu-pro-val-glu-tyr-leu-gln-val-pro-ser-pro-ser-m t-
      -1  +1
414  GGC-CGT-GAC-ATC-AAG}-GTC-CAA-TTC-CAA-AGT-GGT-GGT-GCC-AAC-TCG-CCC-GCC-CTG-TAC-CTG-
 19  gly-arg-asp-ile-lys -val-gln-phe-gln-ser-gly-gly-ala-asn-ser-pro-ala-leu-tyr-l u-
                         ↓ 17

474  CTC-GAC-GGC-CTG-CGC-GCG-CAG-GAC-GAC-TTC-AGC-GGC-TGG-GAC-ATC-AAC-ACC-CCG-GCG-TTC-
 39  l u-asp-gly-leu-arg-ala-gln-asp-asp-phe-ser-gly-trp-asp-ile-asn-thr-pro-ala-ph -

534  GAG-TGG-TAC-GAC-CAG-TCG-GGC-CTG-TCG-GTG-GTC-ATG-CCG-GTG-GGT-GGC-CAG-TCA-AGC-TTC-
 59  glu-trp-tyr-asp-gln-ser-gly-leu-ser-val-val-met-pro-val-gly-gly-gln-ser-ser-phe-

594  TAC-TCC-GAC-TGG-TAC-CAG-CCC-GCC-TGC-CGC-AAG-GCC-GGT-TGC-CAG-(ACT-TAC-AAG-TGG-GAG-
 79  tyr-ser-asp-trp-tyr-gln-pro-ala-cys-arg-lys-ala-gly-cys-gln- thr-tyr-lys-trp-glu-
```

Figure 4a

```
654  ACC-TTC-CTG-ACC-AGC-GAG-CTG-CCG-GGG-TGG-CTG-CAG-GCC-AAC-AGG-CAC-GTC-AAG-CCC-ACC-
 99  thr-phe-leu-thr-ser-glu-leu-pro-gly-trp-leu-gln-ala-asn-arg-his-val-lys-pro-thr-

714  GGA-AGC-GCC-GTC-GTC-GGT-CTT-TCG-ATG-GCT-GCT-TCT-TCG-GCG-CTG-ACG-CTG-GCG-ATC-TAT-
119  gly-ser-ala-val-val-gly-leu-ser-met-ala-ala-ser-ser-ala-leu-thr-leu-ala-il -tyr-

774  CAC-CCC-CAG-CAG-TTC-GTC-TAC-GCG-GGA-GCG-ATG-TCG-GGC-CTG-TTG-GAC-CCC-TCC-CAG-GCG-
139  his-pro-gln-gln-phe-val-tyr-ala-gly-ala-met-ser-gly-leu-leu-asp-pro-ser-gln-ala-

834  ATG-GGT-CCC-ACC-CTG-ATC-GGC-CTG-GCG-ATG-GGT-GAC-GCT-GGC-GGC-TAC-AAG-GCC-TCC-GAC-
159  m t-gly-pro-thr-leu-ile-gly-leu-ala-met-gly-asp-ala-gly-gly-tyr-lys-ala-ser-asp-

894  ATG-TGG-GGC-CCG-AAG-GAG-GAC-CCG-GCG-TGG-CAG-CGC-AAC-GAC-CCG-CTG-TTG-AAC-GTC-GGG-
179  met-trp-gly-pro-lys-glu-asp-pro-ala-trp-gln-arg-asn-asp-pro-leu-leu-asn-val-gly-

954  AAG-CTG-ATC-GCC-AAC-AAC-ACC-CGC-GTC-TGG-GTG-TAC-TGC-GGC-AAC-GGC-AAG-CCG-TCG-GAT-
199  lys-leu-ile-ala-asn-asn-thr-arg-val-trp-val-tyr-cys-gly-asn-gly-lys-pro-ser-asp-
                 ↓ 24
1014 CTG-GGT-GGC-AAC-AAC-CTG-CCG-GCC-AAG-TTC-CTC-GAG-GGC-TTC-GTG-CGG-ACC-AGC-AAC-ATC-
219  l u-gly-gly-asn-asn-leu-pro-ala-lys-phe-leu-glu-gly-phe-val-arg-thr-ser-asn-il -

1074 AAG-TTC-CAA-GAC-GCC-TAC-AAC-GCC-GGT-GGG- CGC-CAC-AAC-GGC-GTG-TTC-GAC-TTC-CCG-GAC-
239  lys-phe-gln-asp-ala-tyr-asn-ala-gly-gly- arg-his-asn-gly-val-phe-asp-phe-pro-asp-

1134 AGC-GGT-ACG-CAC-AGC-TGG-GAG-TAC-TGG-GGC-GCG-CAG-CTC-AAC-GCT-ATG-AAG-CCC-GAC-CTG-
259  ser-gly-thr-his-ser-trp-glu-tyr-trp-gly-ala-gln-leu-asn-ala-met-lys-pro-asp-l u-
                                                                             1242
1194 CAA-CG -CAC-TGG-GTG-CCA-CGC-CCA-ACA-CCG-GGC-CCG- CCG-CAG-GGC-GCC-TAGCTCCGAACAGACA
279  gln-arg- his-trp- val-pro-arg - pro-thr- pro-gly-pro- pro-gln-gly-ala-TER
                                                                 294
1258 CAACATCTAGCNNCGGTGACCCTTGTGGNNCANATGTTTCCTAAATCCCGTCCCTAGCTCCCGCNGCNNCCGTGTGGTTA
1338 GCTACCTGACNNCATGGGTTT 1358
```

Figure 4b

1 ACT-GCC-GGG-CCC-AGC-GCC-TGC-AGT-CTG-ACC-TAA-TTC-AGG-ATG-CGC-CCA-AAC-ATG-CAT-GGA-
61 TGC-GTT-GAG-ATG-AGG-ATG-AGG-GAA-GCA-AGA-ATG-CAG-CTT-GTT-GAC-AGG-GTT-CGT-GGC-GCC-
MET-GLN-LEU-VAL-ASP-ARG-VAL-ARG-GLY-ALA-
(-43)

121 GTC-ACG-GGT-ATG-TCG-CGT-CGA-CTC-GTG-GTC-GGG-GCC-GTC-GGC-GCG-GCC-CTA-GTG-TCG-GGT-
-33 VAL-THR-GLY-MET-SER-ARG-ARG-LEU-VAL-VAL-GLY-ALA-VAL-GLY-ALA-ALA-LEU-VAL-SER-GLY-

181 CTG-GTC-GGC-GCC-GTC-GGT-GGC-ACG-GCG-ACC-GCG-GGG-GCA-TTT-TCC-CGG-CCG-GGC-TTG-CCG-
-13 LEU-VAL-GLY-ALA-VAL-GLY-GLY-THR-ALA-THR-ALA-GLY-ALA-phe-ser-arg-pro-gly-leu-pro-
+1

241 GTG-GAG-TAC-CTG-CAG-GTG-CCG-TCG-CCG-TCG-ATG-GGC-CGT-GAC-ATC-AAG-GTC-CAA-TTC-CAA-
8 val-glu-tyr-leu-gln-val-pro-ser-pro-ser-met-gly-arg-asp-ile-lys-val-gln-phe-gln-
↓17

301 AGT-GGT-GGT-GCC-AAC-TCG-CCC-GCC-CTG-TAC-CTG-CTC-GAC-GGC-CTG-CGC-GCG-CAG-GAC-GAC-
28 ser-gly-gly-ala-asn-ser-pro-ala-leu-tyr-leu-leu-asp-gly-leu-arg-ala-gln-asp-asp-

361 TTC-AGC-GGC-TGG-GAC-ATC-AAC-ACC-CCG-GCG-TTC-GAG-TGG-TAC-GAC-CAG-TCG-GGC-CTG-TCG-
48 phe-ser-gly-trp-asp-ile-asn-thr-pro-ala-phe-glu-trp-tyr-asp-gln-ser-gly-leu-ser-

421 GTG-GTC-ATG-CCG-GTG-GGT-GGC-CAG-TCA-AGC-TTC-TAC-TCC-GAC-TGG-TAC-CAG-CCC-GCC-TGC-
68 val-val-met-pro-val-gly-gly-gln-ser-ser-phe-tyr-ser-asp-trp-tyr-gln-pro-ala-cys-

481 GGC-AAG-GCC-GGT-TGC-CAG-ACT-TAC-AAG-TGG-GAG-ACC-TTC-CTG-ACC-AGC-GAG-CTG-CCG-GGG-
88 gly-lys-ala-gly-cys-gln-thr-tyr-lys-trp-glu-thr-phe-leu-thr-ser-glu-leu-pro-gly-

Figure 5

```
541  TGG-CTG-CAG-GCC-AAC-AGG-CAC-GTC-AAG-CCC-ACC-GGA-AGC-GCC-GTC-GTC-GGT-CTT-TCG-ATG-
108  trp-leu-gln-ala-asn-arg-his-val-lys-pro-thr-gly-ser-ala-val-val-gly-leu-ser-met-

601  GCT-GCT-TCT-TCG-GCG-CTG-ACG-CTG-GCG-ATC-TAT-CAC-CCC-CAG-CAG-TTC-GTC-TAC-GCG-GGA-
128  ala-ala-ser-ser-ala-leu-thr-leu-ala-ile-tyr-his-pro-gln-gln-phe-val-tyr-ala-gly-

661  GCG-ATG-TCG-GGC-CTG-TTG-GAC-CCC-TCC-CAG-GCG-ATG-GGT-CCC-ACC-CTG-ATC-GGC-CTG-GCG-
148  ala-met-ser-gly-leu-leu-asp-pro-ser-gln-ala-met-gly-pro-thr-leu-ile-gly-leu-ala-

721  ATG-GGT-GAC-GCT-GGC-GGC-TAC-AAG-GCC-TCC-GAC-ATG-TGG-GGC-CCG-AAG-GAG-GAC-CCG-GCG-
168  met-gly-asp-ala-gly-gly-tyr-lys-ala-ser-asp-met-trp-gly-pro-lys-glu-asp-pro-ala-

781  TGG-CAG-CGC-AAC-GAC-CCG-CTG-TTG-AAC-GTC-GGG-AAG-CTG-ATC-GCC-AAC-AAC-ACC-CGC-GTC-
188  trp-gln-arg-asn-asp-pro-leu-leu-asn-val-gly-lys-leu-ile-ala-asn-asn-thr-arg-val-
                                                           24
                                                           ↓
841  TGG-GTG-TAC-TGC-GGC-AAC-GGC-AAG-CCG-TCG-GAT-CTG-GGT-GGC-AAC-AAC-CTG-CCG-GCC-AAG-
208  trp-val-tyr-cys-gly-asn-gly-lys-pro-ser-asp-leu-gly-gly-asn-asn-leu-pro-ala-lys-

901  TTC-CTC-GAG-GGC-TTC-GTG-CGG-ACC-AGC-AAC-ATC-AAG-TTC-CAA-GAC-GCC-TAC-AAC-GCC-GGT-
228  phe-leu-glu-gly-phe-val-arg-thr-ser-asn-ile-lys-phe-gln-asp-ala-tyr-asn-ala-gly-

961  GGC-GGC-CAC-AAC-GGC-GTG-TTC-GAC-TTC-CCG-GAC-AGC-GGT-ACG-CAC-AGC-TGG-GAG-TAC-TGG-
248  gly-gly-his-asn-gly-val-phe-asp-phe-pro-asp-ser-gly-thr-his-ser-trp-glu-tyr-trp-
```

Figure 5 (con't)

```
1021  GG[C]-GCG-CAG-CTC-AAC-GCT-ATG-AAG-CCC-GAC-CTG-CAA-CGG-GCA-CTG-GGT-GCC-ACG-CCC-AAC-
268   gly-ala-gln-leu-asn-ala-met-lys-pro-asp-leu-gln-arg-ala-leu-gly-ala-thr-pro-asn-
                                                 (1104)
1081  ACC-GGG-CCC-GCG-CCC-CAG-GGC-GCC-TAG-CTC-CGA-ACA-GAC-ACA-ACA-TCT-AGC-GGC-GGT-GAC-
288   thr-gly-pro-ala-pro-gln-gly-ala-TER
                                (295)

1141  CCT-TGT-GGT-CGC-CGC-CGT-AGA-TGT-TTC-CTA-AAT-CCC-GTC-CCT-AGC-TCC-CGC-CGC-GGG-CCG-
1201  TGT-GGT-TAG-CTA-CCT-GAC-GGG-CTA-GGG-GTT-GGC-CGG-GGC-GGT-TGA-CGC-CGG-GTG-CAC-ACA-
1261  GCC-TAC-ACG-AAC-GGA-AGG-TGG-ACA-CAT-GAA-GGG-TCG-GTC
                                                  (1299)
```

Figure 5 (con't)

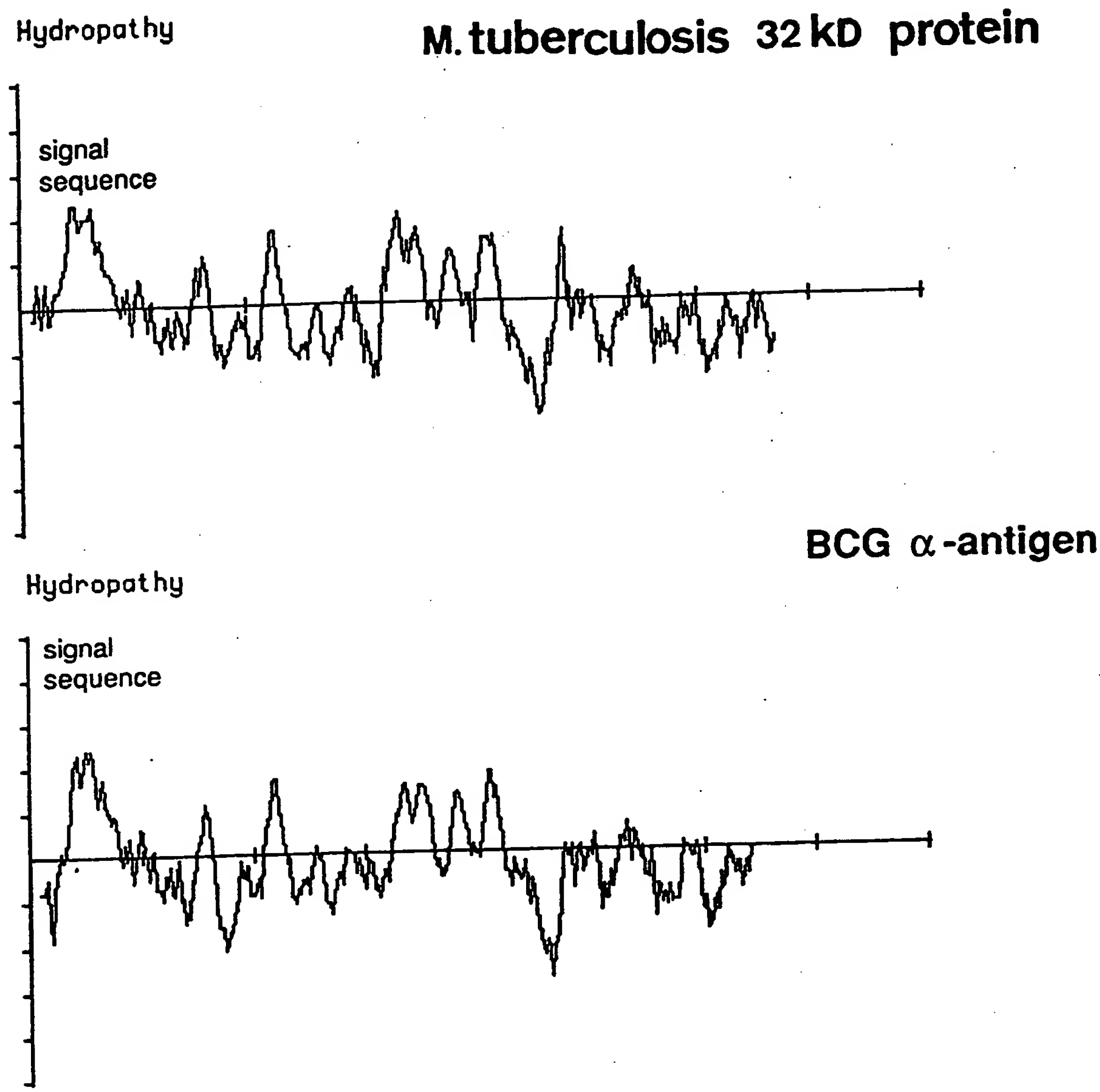
Hydropathy
M. tuberculosis 32 kD protein
signal sequence
BCG α-antigen
Hydropathy
signal sequence

Fig. 6

```
              10         20         30         40         50         60
M.tub.  VDRVRGAVTGMSRRLVVGAVGAALVSGLVGAVGGTATAGAFSRPGLPVEYLQVPSPSMGR
        .. :.   . . .:::.:...:....:::: .:::.::::::::::::::::::::::::
BCG     MTDVSRKIRAWGRRLMIGTAAAVVLPGLVGLAGGAATAGAFSRPGLPVEYLQVPSPSMGR
                10         20         30         40         50         60

              70         80         90        100        110        120
        DIKVQFQSGGANSPALYLLDGLRAQDDFSGWDINTPAFEWYDQSGLSVVMPVGGQSSFYS
        ::::::::::.::::.:::::::::::..::::::::::: :::::.:::::::::::::
        DIKVQFQSGGNNSPAVYLLDGLRAQDDYNGWDINTPAFEWYYQSGLSIVMPVGGQSSFYS
                70         80         90        100        110        120

             130        140        150        160          170        180
        DWYQPACGKAGCQTYKWETFLTSELPGWLQANRHVKPTG--SAVVGLSMAASSALTLAIY
        ::: ::::::::::::::::.:::::: :: ::: :::::  ::..::::.:::..:: :
        DWYSPACGKAGCQTYKWETLLTSELPQWLSANRAVKPTGSPSAAIGLSMAGSSAMILAAY
               130        140        150        160        170        180

              190        200        210        220        230        240
        HPQQFVYAGAMSGLLDPSQAMGPTLIGLAMGDAGGYKASDMWGPKEDPAWQRNDPLLNVG
        :::::.:::..:.:::::::.::  :::::::::::::::.:::::..::::.::::  ..
        HPQQFIYAGSLSALLDPSQGMG--LIGLAMGDAGGYKAADMWGPSSDPAWERNDPTQQIP
               190        200          210        220        230
```

Fig. 7

```
    250       260       270       280       290       300
KLIANNTRVWVYCGNGKPSDLGGNNLPAKFLEGFVRTSNIKFQDAYNAGGGHNGVFDFPD
::.::::::.:::::::.:..:::.:.::.:::.:::.::.::::::...::::.::.::
KLVANNTRLWVYCGNGTPNELGGANIPAEFLENFVRSSNLKFQDAYKPAGGHNAVFNFPP
240       250       260       270       280       290

    310       320
SGTHSWEYWGAQLNAMKPDLQRALGA
.:::::::::::::::: :::..::X
NGTHSWEYWGAQLNAMKGDLQSSLGA
300       310       320
```

Fig. 7 (con't)

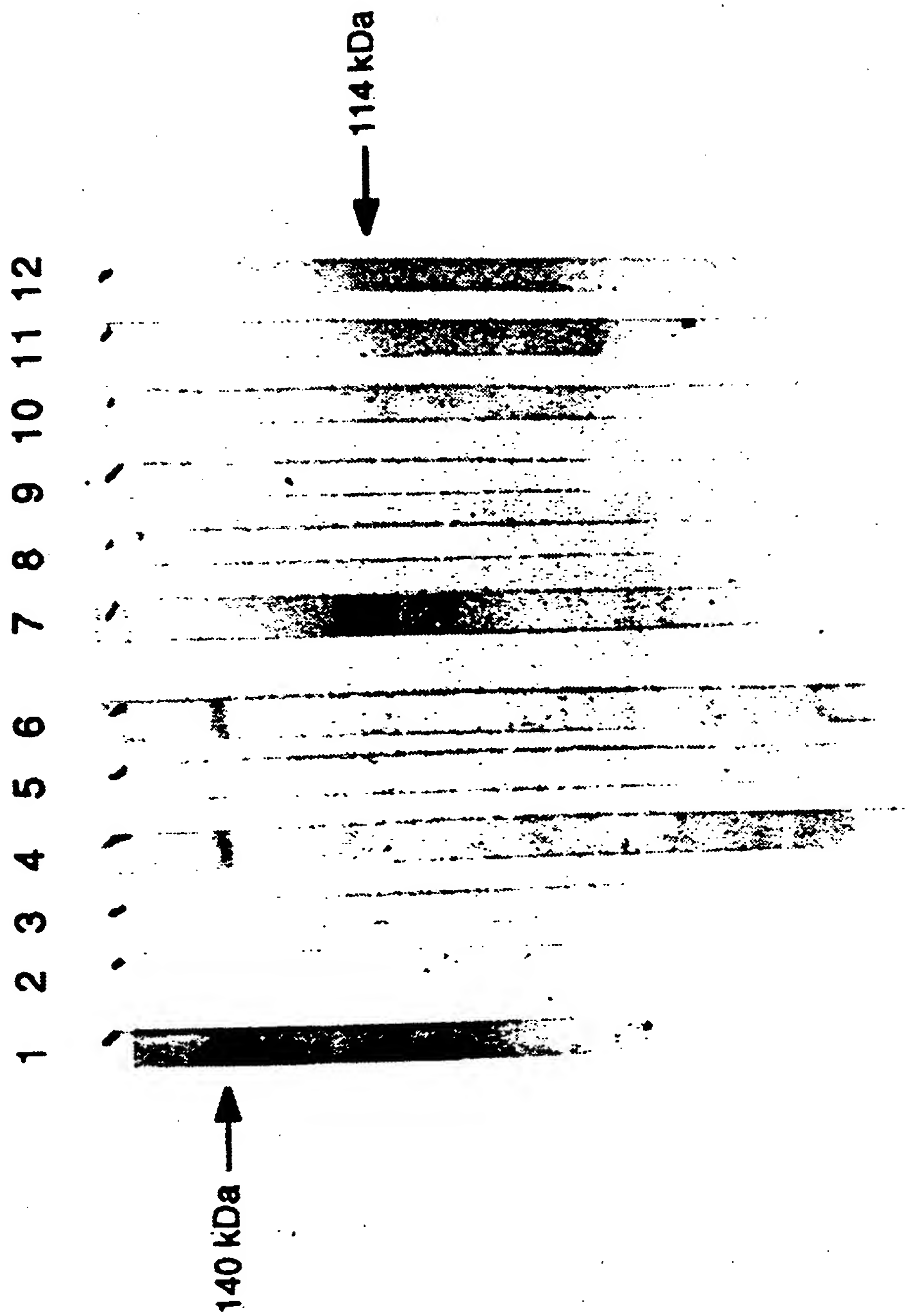

Figure 8

Figur 9b

```
                          P2
270  CTTCAGCGGCTGGGAC ATCAACACCCCGGCGTTCGAGTGGTAC GACCAGTCGG
267  CTTCAGCGGCTGGGAC ATCAACACCCCGGCGTTCGAGTGGTAC GACCAGTCGG
261  CTACAACGGCTGGGAT ATCAACACCCCGGCGTTCGAGTGGTAC TACCAGTCGG

323  GCCTGTCGGTGGTCATGCCGGTGGGTGGCCAGTCAAGCTTCTACTCCGACTGGTA
320  GCCTGTCGGTGGTCATGCCGGTGGGTGGCCAGTCAAGCTTCTACTCCGACTGGTA
314  GACTGTCGATAGTCATGCCGGTCGGCGGGCAGTCCAGCTTCTACAGCGACTGGTA

                                   P3                         P4
378  CCAGCCCGCCTGCGGCAAGGCCGGT TGCCAGACTTACAAGTGGGA GACCT TC
375  CCAGCCCGCCTGCCGCAAGGCCGGT TGCCAGACTTACAAGTGGGA GACCT TC
369  CAGCCCGGCCTGCGGTAAGGCTGGC TGCCAGACTTACAAGTGGGA AACCC TC

430  CTGACCAGCGAGCTGCCG GGGTGGCTGCAGGCCAACAGGCACGTCAAGCCCACC
427  CTGACCAGCGAGCTGCCG GGGTGGCTGCAGGCCAACAGGCACGTCAAGCCCACC
421  CTGACCAGCGAGCTGCCG CAATGGTTGTCCGCCAACAGGGCCGTGAAGCCCACC

                                                 PROBE REGION C
484  GGAAGCGCCGTCGTCGGTCTTTCGATGGCTGCTTCTTCG GCGCTGACGCTGGCG
481  GGAAGCGCCGTCGTCGGTCTTTCGATGGCTGCTTCTTCG GCGCTGACGCTGGCG
475  GGCAGCGCTGCAATCGGCTTGTCGATGGCCGGCTCGTCG GCAATGATCTTGGCC
```

SUBSTITUTE SHEET

Figure 9c

```
538  ATCTATC ACCCCCAGCAGTTCGTCTACGCGGGAGCGATGTCGGCCTGTTGGAC
535  ATCTATC ACCCCCAGCAGTTCGTCTACGCGGGAGCGATGTCGGCCTGTTGGAC
529  GCCTACC ACCCCCAGCAGTTCATCTACGCCGGCTCGCTGTCGGCCCTGCTGGAC

                                P5
592  CCCTCCCAGGCGATGGGTCCCAC CCTGATCGGCCTGGCGATGGGTGACGC TGG
589  CCCTCCCAGGCGATGGGTCCCAC CCTGATCGGCCTGGCGATGGGTGACGC TGG
583  CCCTCTCAGGGGATGGG       CCTGATCGGCCTCGCGATGGGTGACGC CGG

645  CGGCTACAAGGCCTCCGACATGTGGGGCCCGAAGGAGGACCCGGCGTGGCAGCGC
642  CGGCTACAAGGCCTCCGACATGTGGGGCCCGAAGGAGGACCCGGCGTGGCAGCGC
631  CGGTTACAAGGCCGCAGACATGTGGGGTCCCTCGAGTGACCCGGCATGGGAGCGC

            PROBE REGION D
700  AACGAC CCGCTGTTGAACGTCGGGAAG CTGATCGCCAACAACACCCGCGTCTG
697  AACGAC CCGCTGTTGAACGTCGGGAAG CTGATCGCCAACAACACCCGCGTCTG
686  AACGAC CCTACGCAGCAGATCCCCAAG CTGGTCGCAAACAACACCCGGCTATG

                        PROBE REGION E
753  GGTGTACTGCGGCAACGGC AAGCCGTCGGATCTGGGTGGCAAC AACCTGCCGG
750  GGTGTACTGCGGCAACGGC AAGCCGTCGGATCTGGGTGGCAAC AACCTGCCGG
739  GGTTTATTGCGGGAACGGC ACCCCGAACGAGTTGGGCGGTGCC AACATACCCG
```

Figure 9d

Figure 9e

```
806   CCAAGTTCCTCGAGGGCTTCGTGCGGACCAGCAACATCAAGTTCCAAGACGCCTA
803   CCAAGTTCCTCGAGGGCTTCGTGCGGACCAGCAACATCAAGTTCCAAGACGCCTA
792   CCGAGTTCTTGGAGAACTTCGTTCGTAGCAGCAACCTGAAGTTCCAGGATGCGTA

                                                      P6
861   CAACGCCGGTGGCGGCCACAACGGCGTGTTCGACTTCCCGGACAGCGGT ACGCA
858   CAACGCCGGTGGGCGCCACAACGGCGTGTTCGACTTCCCGGACAGCGGT ACGCA
847   CAAGCCCGCGGGCGGGCACAACGCCGTGTTCAACTTCCCGCCCAACGGC ACGCA

915   CAGCTGGGAGTACTGGGGCGC GCAGCTCAACGCTATGAAGCCCGACCTGCA AC
912   CAGCTGGGAGTACTGGGGCGC GCAGCTCAACGCTATGAAGCCCGACCTGCA AC
901   CAGCTGGGAGTACTGGGGCGC TCAGCTCAACGCCATGAAGGGTGACCTGCAGAG

      PROBE REGION F
968   GGGCACTGGGTGCCACGCCCAACACCGGGCCCGCGCCCCAGGG CGCCTAG
965    GGCACTGGGTGCCACGCCCAACACCGGGCC   CGCCGCAGGG CGCCTAG
955    TTCGTTAGGCGCC GGCTGA
```

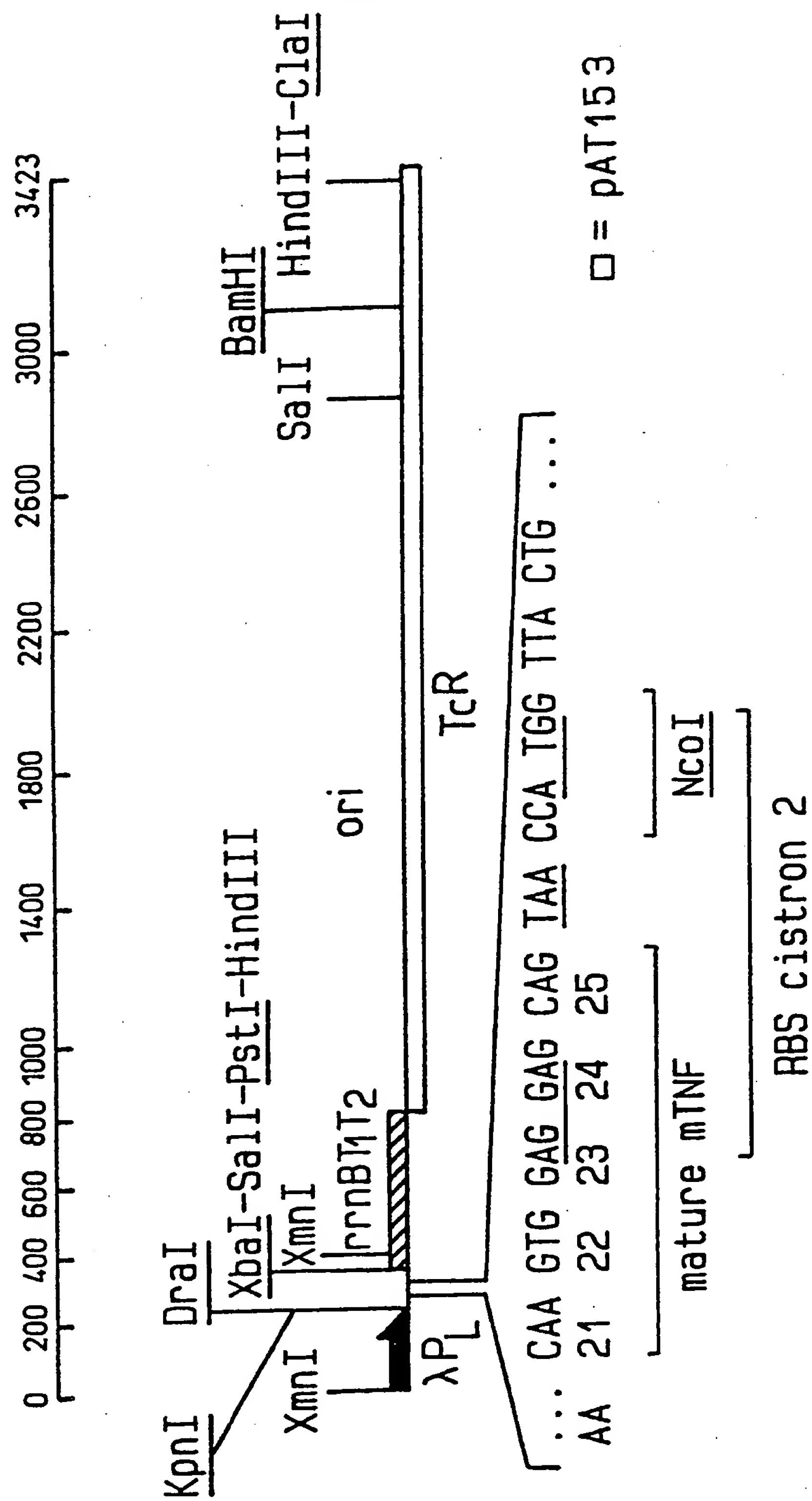
0
200
400
600
800
1000
1400
1800
2200
2600
3000
3423
KpnI
XmnI
DraI
XbaI-SalI-PstI-HindIII
XmnI
rrnBT1T2
ori
SalI
BamHI
HindIII-ClaI
TcR
λPL
□ = pAT153
... CAA GTG GAG GAG CAG TAA CCA TGG TTA CTG ...
AA 21 22 23 24 25
mature mTNF
NcoI
RBS cistron 2

FIG. 10a

From: PIGRI

Fig. 10b

```
      3           9          15          21          27          33          39          45
      |           |           |           |           |           |           |           |
  1 TTC CGG GGA TCT CTC ACC TAC CAA ACA ATG CCC CCC TGC AAA AAA
    AAG GCC CCT AGA GAG TGG ATG GTT TGT TAC GGG GGG ACG TTT TTT

 46 TAA ATT CAT ATA AAA AAC ATA CAG ATA ACC ATC TGC GGT GAT AAA
    ATT TAA GTA TAT TTT TTG TAT GTC TAT TGG TAG ACG CCA CTA TTT

 91 TTA TCT CTG GCG GTG TTG ACA TAA ATA CCA CTG GCG GTG ATA CTG
    AAT AGA GAC CGC CAC AAC TGT ATT TAT GGT GAC CGC CAC TAT GAC

136 AGC ACA TCA GCA GGA CGC ACT GAC CAC CAT GAA GGT GAC GCT CTT
    TCG TGT AGT CGT CCT GCG TGA CTG GTG GTA CTT CCA CTG CGA GAA

181 AAA AAT TAA GCC CTG AAG AAG GGC AGG GGT ACC AGG AGG TTT AAA
    TTT TTA ATT CGG GAC TTC TTC CCG TCC CCA TGG TCC TCC AAA TTT

226 TCA TGG TAA GAT CAA GTA GTC AAA ATT CGA GTG ACA AGC CTG TAG
    AGT ACC ATT CTA GTT CAT CAG TTT TAA GCT CAC TGT TCG GAC ATC

271 CCC ACG TCG TAG CAA ACC ACC AAG TGG AGG AGC AGT AAC CAT GGT
    GGG TGC AGC ATC GTT TGG TGG TTC ACC TCC TCG TCA TTG GTA CCA

316 TAC TGG AGA AGG GGG ACC AAC TCA GCG CTG AGG TCA ATC TGC CCA
    ATG ACC TCT TCC CCC TGG TTG AGT CGC GAC TCC AGT TAG ACG GGT
```

Fig. 10b (Con't)

```
361 AGT CTA GAG TCG ACC TGC AGC CCA AGC TTG GCT GTT TTG GCG GAT
    TCA GAT CTC AGC TGG ACG TCG GGT TCG AAC CGA CAA AAC CGC CTA

406 GAG AGA AGA TTT TCA GCC TGA TAC AGA TTA AAT CAG AAC GCA GAA
    CTC TCT TCT AAA AGT CGG ACT ATG TCT AAT TTA GTC TTG CGT CTT

451 GCG GTC TGA TAA AAC AGA ATT TGC CTG GCG GCA GTA GCG CGG TGG
    CGC CAG ACT ATT TTG TCT TAA ACG GAC CGC CGT CAT CGC GCC ACC

496 TCC CAC CTG ACC CCA TGC CGA ACT CAG AAG TGA AAC GCC GTA GCG
    AGG GTG GAC TGG GGT ACG GCT TGA GTC TTC ACT TTG CGG CAT CGC

541 CCG ATG GTA GTG TGG GGT CTC CCC ATG CGA GAG TAG GGA ACT GCC
    GGC TAC CAT CAC ACC CCA GAG GGG TAC GCT CTC ATC CCT TGA CGG

586 AGG CAT CAA ATA AAA CGA AAG GCT CAG TCG AAA GAC TGG GCC TTT
    TCC GTA GTT TAT TTT GCT TTC CGA GTC AGC TTT CTG ACC CGG AAA

631 CGT TTT ATC TGT TGT TTG TCG GTG AAC GCT CTC CTG AGT AGG ACA
    GCA AAA TAG ACA ACA AAC AGC CAC TTG CGA GAG GAC TCA TCC TGT

676 AAT CCG CCG GGA GCG GAT TTG AAC GTT GCG AAG CAA CGG CCC GGA
    TTA GGC GGC CCT CGC CTA AAC TTG CAA CGC TTC GTT GCC GGG CCT

721 GGG TGG CGG GCA GGA CGC CCG CCA TAA ACT GCC AGG CAT CAA ATT
    CCC ACC GCC CGT CCT GCG GGC GGT ATT TGA CGG TCC GTA GTT TAA
```

Fig. 10b (Con't)

```
766  AAG CAG AAG GCC ATC CTG ACG GAT GGC CTT TTT GCG TTT CTA CAA
     TTC GTC TTC CGG TAG GAC TGC CTA CCG GAA AAA CGC AAA GAT GTT

811  ACT CTT TTG TTT ATT TTT CTA AAT ACA TTC AAA TAT GTA TCC GCT
     TGA GAA AAC AAA TAA AAA GAT TTA TGT AAG TTT ATA CAT AGG CGA

856  CAT GAG ACA ATA ACC CTG ATA AAT GCT TCA ATA ATA AAA GGA TCT
     GTA CTC TGT TAT TGG GAC TAT TTA CGA AGT TAT TAT TTT CCT AGA

901  AGG TGA AGA TCC TTT TTG ATA ATC TCA TGA CCA AAA TCC CTT AAC
     TCC ACT TCT AGG AAA AAC TAT TAG AGT ACT GGT TTT AGG GAA TTG

946  GTG AGT TTT CGT TCC ACT GAG CGT CAG ACC CCG TAG AAA AGA TCA
     CAC TCA AAA GCA AGG TGA CTC GCA GTC TGG GGC ATC TTT TCT AGT

991  AAG GAT CTT CTT GAG ATC CTT TTT TTC TGC GCG TAA TCT GCT GCT
     TTC CTA GAA GAA CTC TAG GAA AAA AAG ACG CGC ATT AGA CGA CGA

1036 TGC AAA CAA AAA AAC CAC CGC TAC CAG CGG TGG TTT GTT TGC CGG
     ACG TTT GTT TTT TTG GTG GCG ATG GTC GCC ACC AAA CAA ACG GCC
```

Fig. 10b (Con't)

```
1081 ATC AAG AGC TAC CAA CTC TTT TTC CGA AGG TAA CTG GCT TCA GCA
     TAG TTC TCG ATG GTT GAG AAA AAG GCT TCC ATT GAC CGA AGT CGT

1126 GAG CGC AGA TAC CAA ATA CTG TCC TTC TAG TGT AGC CGT AGT TAG
     CTC GCG TCT ATG GTT TAT GAC AGG AAG ATC ACA TCG GCA TCA ATC

1171 GCC ACC ACT TCA AGA ACT CTG TAG CAC CGC CTA CAT ACC TCG CTC
     CGG TGG TGA AGT TCT TGA GAC ATC GTG GCG GAT GTA TGG AGC GAG

1216 TGC TAA TCC TGT TAC CAG TGG CTG CTG CCA GTG GCG ATA AGT CGT
     ACG ATT AGG ACA ATG GTC ACC GAC GAC GGT CAC CGC TAT TCA GCA

1261 GTC TTA CCG GGT TGG ACT CAA GAC GAT AGT TAC CGG ATA AGG CGC
     CAG AAT GGC CCA ACC TGA GTT CTG CTA TCA ATG GCC TAT TCC GCG

1306 AGC GGT CGG GCT GAA CGG GGG GTT CGT GCA CAC AGC CCA GCT TGG
     TCG CCA GCC CGA CTT GCC CCC CAA GCA CGT GTG TCG GGT CGA ACC

1351 AGC GAA CGA CCT ACA CCG AAC TGA GAT ACC TAC AGC GTG AGC ATT
     TCG CTT GCT GGA TGT GGC TTG ACT CTA TGG ATG TCG CAC TCG TAA
```

Fig. 10b (Con't)

```
1396 GAG AAA GCG CCA CGC TTC CCG AAG GGA GAA AGG CGG ACA GGT ATC
     CTC TTT CGC GGT GCG AAG GGC TTC CCT CTT TCC GCC TGT CCA TAG

1441 CGG TAA GCG GCA GGG TCG GAA CAG GAG AGC GCA CGA GGG AGC TTC
     GCC ATT CGC CGT CCC AGC CTT GTC CTC TCG CGT GCT CCC TCG AAG

1486 CAG GGG GAA ACG CCT GGT ATC TTT ATA GTC CTG TCG GGT TTC GCC
     GTC CCC CTT TGC GGA CCA TAG AAA TAT CAG GAC AGC CCA AAG CGG

1531 ACC TCT GAC TTG AGC GTC GAT TTT TGT GAT GCT CGT CAG GGG GGC
     TGG AGA CTG AAC TCG CAG CTA AAA ACA CTA CGA GCA GTC CCC CCG

1576 GGA GCC TAT GGA AAA ACG CCA GCA ACG CGG CCT TTT TAC GGT TCC
     CCT CGG ATA CCT TTT TGC GGT CGT TGC GCC GGA AAA ATG CCA AGG
```

Fig. 10b (Con't)

```
1621 TGG CCT TTT GCT GGC CTT TTG CTC ACA TGT TCT TTC CTG CGT TAT
     ACC GGA AAA CGA CCG GAA AAC GAG TGT ACA AGA AAG GAC GCA ATA

1666 CCC CTG ATT CTG TGG ATA ACC GTA TTA CCG CCT TTG AGT GAG CTG
     GGG GAC TAA GAC ACC TAT TGG CAT AAT GGC GGA AAC TCA CTC GAC

1711 ATA CCG CTC GCC GCA GCC GAA CGA CCG AGC GCA GCG AGT CAG TGA
     TAT GGC GAG CGG CGT CGG CTT GCT GGC TCG CGT CGC TCA GTC ACT

1756 GCG AGG AAG CGG AAG AGC GCT GAC TTC CGC GTT TCC AGA CTT TAC
     CGC TCC TTC GCC TTC TCG CGA CTG AAG GCG CAA AGG TCT GAA ATG

1801 GAA ACA CGG AAA CCG AAG ACC ATT CAT GTT GTT GCT CAG GTC GCA
     CTT TGT GCC TTT GGC TTC TGG TAA GTA CAA CAA CGA GTC CAG CGT

1846 GAC GTT TTG CAG CAG CAG TCG CTT CAC GTT CGC TCG CGT ATC GGT
     CTG CAA AAC GTC GTC GTC AGC GAA GTG CAA GCG AGC GCA TAG CCA

1891 GAT TCA TTC TGC TAA CCA GTA AGG CAA CCC CGC CAG CCT AGC CGG
     CTA AGT AAG ACG ATT GGT CAT TCC GTT GGG GCG GTC GGA TCG GCC
```

Fig. 10b (Con't)

```
1936 GTC CTC AAC GAC AGG AGC ACG ATC ATG CGC ACC CGT GGC CAG GAC
     CAG GAG TTG CTG TCC TCG TGC TAG TAC GCG TGG GCA CCG GTC CTG

1981 CCA ACG CTG CCC GAG ATG CGC CGC GTG CGG CTG CTG GAG ATG GCG
     GGT TGC GAC GGG CTC TAC GCG GCG CAC GCC GAC GAC CTC TAC CGC

2026 GAC GCG ATG GAT ATG TTC TGC CAA GGG TTG GTT TGC GCA TTC ACA
     CTG CGC TAC CTA TAC AAG ACG GTT CCC AAC CAA ACG CGT AAG TGT

2071 GTT CTC CGC AAG AAT TGA TTG GCT CCA ATT CTT GGA GTG GTG AAT
     CAA GAG GCG TTC TTA ACT AAC CGA GGT TAA GAA CCT CAC CAC TTA

2116 CCG TTA GCG AGG TGC CGC CGG CTT CCA TTC AGG TCG AGG TGG CCC
     GGC AAT CGC TCC ACG GCG GCC GAA GGT AAG TCC AGC TCC ACC GGG

2161 GGC TCC ATG CAC CGC GAC GCA ACG CGG GGA GGC AGA CAA GGT ATA
     CCG AGG TAC GTG GCG CTG CGT TGC GCC CCT CCG TCT GTT CCA TAT

2206 GGG CGG CGC CTA CAA TCC ATG CCA ACC CGT TCC ATG TGC TCG CCG
     CCC GCC GCG GAT GTT AGG TAC GGT TGG GCA AGG TAC ACG AGC GGC
```

Fig. 10b (Con't)

```
2251 AGG CGG CAT AAA TCG CCG TGA CGA TCA GCG GTC CAG TGA TCG AAG
     TCC GCC GTA TTT AGC GGC ACT GCT AGT CGC CAG GTC ACT AGC TTC

2296 TTA GGC TGG TAA GAG CCG CGA GCG ATC CTT GAA GCT GTC CCT GAT
     AAT CCG ACC ATT CTC GGC GCT CGC TAG GAA CTT CGA CAG GGA CTA

2341 GGT CGT CAT CTA CCT GCC TGG ACA GCA TGG CCT GCA ACG CGG GCA
     CCA GCA GTA GAT GGA CGG ACC TGT CGT ACC GGA CGT TGC GCC CGT

2386 TCC CGA TGC CGC CGG AAG CGA GAA GAA TCA TAA TGG GGA AGG CCA
     AGG GCT ACG GCG GCC TTC GCT CTT CTT AGT ATT ACC CCT TCC GGT

2431 TCC AGC CTC GCG TCG CGA ACG CCA GCA AGA CGT AGC CCA GCG CGT
     AGG TCG GAG CGC AGC GCT TGC GGT CGT TCT GCA TCG GGT CGC GCA
```

Fig. 10b (Con't)

```
2476 CGG CCG CCA TGC CGG CGA TAA TGG CCT GCT TCT CGC CGA AAC GTT
     GCC GGC GGT ACG GCC GCT ATT ACC GGA CGA AGA GCG GCT TTG CAA

2521 TGG TGG CGG GAC CAG TGA CGA AGG CTT GAG CGA GGG CGT GCA AGA
     ACC ACC GCC CTG GTC ACT GCT TCC GAA CTC GCT CCC GCA CGT TCT

2566 TTC CGA ATA CCG CAA GCG ACA GGC CGA TCA TCG TCG CGC TCC AGC
     AAG GCT TAT GGC GTT CGC TGT CCG GCT AGT AGC AGC GCG AGG TCG

2611 GAA AGC GGT CCT CGC CGA AAA TGA CCC AGA GCG CTG CCG GCA CCT
     CTT TCG CCA GGA GCG GCT TTT ACT GGG TCT CGC GAC GGC CGT GGA

2656 GTC CTA CGA GTT GCA TGA TAA AGA AGA CAG TCA TAA GTG CGG CGA
     CAG GAT GCT CAA CGT ACT ATT TCT TCT GTC AGT ATT CAC GCC GCT

2701 CGA TAG TCA TGC CCC GCG CCC ACC GGA AGG AGC TGA CTG GGT TGA
     GCT ATC AGT ACG GGG CGC GGG TGG CCT TCC TCG ACT GAC CCA ACT

2746 AGG CTC TCA AGG GCA TCG GTC GAC GCT CTC CCT TAT GCG ACT CCT
     TCC GAG AGT TCC CGT AGC CAG CTG CGA GAG GGA ATA CGC TGA GGA
```

Fig. 10b (Con't)

```
2791 GCA TTA GGA AGC AGC CCA GTA GTA GGT TGA GGC CGT TGA GCA CCG
     CGT AAT CCT TCG TCG GGT CAT CAT CCA ACT CCG GCA ACT CGT GGC

2836 CCG CCG CAA GGA ATG GTG CAT GCA AGG AGA TGG CGC CCA ACA GTC
     GGC GGC GTT CCT TAC CAC GTA CGT TCC TCT ACC GCG GGT TGT CAG

2881 CCC CGG CCA CGG GGC CTG CCA CCA TAC CCA CGC CGA AAC AAG CGC
     GGG GCC GGT GCC CCG GAC GGT GGT ATG GGT GCG GCT TTG TTC GCG

2926 TCA TGA GCC CGA AGT GGC GAG CCC GAT CTT CCC CAT CGG TGA TGT
     AGT ACT CGG GCT TCA CCG CTC GGG CTA GAA GGG GTA GCC ACT ACA

2971 CGG CGA TAT AGG CGC CAG CAA CCG CAC CTG TGG CGC CGG TGA TGC
     GCC GCT ATA TCC GCG GTC GTT GGC GTG GAC ACC GCG GCC ACT ACG

3016 CGG CCA CGA TGC GTC CGG CGT AGA GGA TCC ACA GGA CGG GTG TGG
     GCC GGT GCT ACG CAG GCC GCA TCT CCT AGG TGT CCT GCC CAC ACC

3061 TCG CCA TGA TCG CGT AGT CGA TAG TGG CTC CAA GTA GCG AAG CGA
     AGC GGT ACT AGC GCA TCA GCT ATC ACC GAG GTT CAT CGC TTC GCT
```

Fig. 10b (Con't)

```
3106 GCA GGA CTG GGC GGC GGC CAA AGC GGT CGG ACA GTG CTC CGA GAA
     CGT CCT GAC CCG CCG CCG GTT TCG CCA GCC TGT CAC GAG GCT CTT

3151 CGG GTG CGC ATA GAA ATT GCA TCA ACG CAT ATA GCG CTA GCA GCA
     GCC CAC GCG TAT CTT TAA CGT AGT TGC GTA TAT CGC GAT CGT CGT

3196 CGC CAT AGT GAC TGG CGA TGC TGT CGG AAT GGA CGA TAT CCC GCA
     GCG GTA TCA CTG ACC GCT ACG ACA GCC TTA CCT GCT ATA GGG CGT

3241 AGA GGC CCG GCA GTA CCG GCA TAA CCA AGC CTA TGC CTA CAG CAT
     TCT CCG GGC CGT CAT GGC CGT ATT GGT TCG GAT ACG GAT GTC GTA

3286 CCA GGG TGA CGG TGC CGA GGA TGA CGA TGA GCG CAT TGT TAG ATT
     GGT CCC ACT GCC ACG GCT CCT ACT GCT ACT CGC GTA ACA ATC TAA
```

Fig. 10b (Con't)

```
3331 TCA TAC ACG GTG CCT GAC TGC GTT AGC AAT TTA ACT GTG ATA AAC
     AGT ATG TGC CAC GGA CTG ACG CAA TCG TTA AAT TGA CAC TAT TTG

3376 TAC CGC ATT AAA GCT TAT CGA TGA TAA GCT GTC AAA CAT GAG AAT
     ATG GCG TAA TTT CGA ATA GCT ACT ATT CGA CAG TTT GTA CTC TTA

3421 TAA
     ATT
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Total number of bases is: 3423.
DNA sequence composition: 839 A; 915 C; 967 G; 702 T;

Sequence name: NIPS0060.

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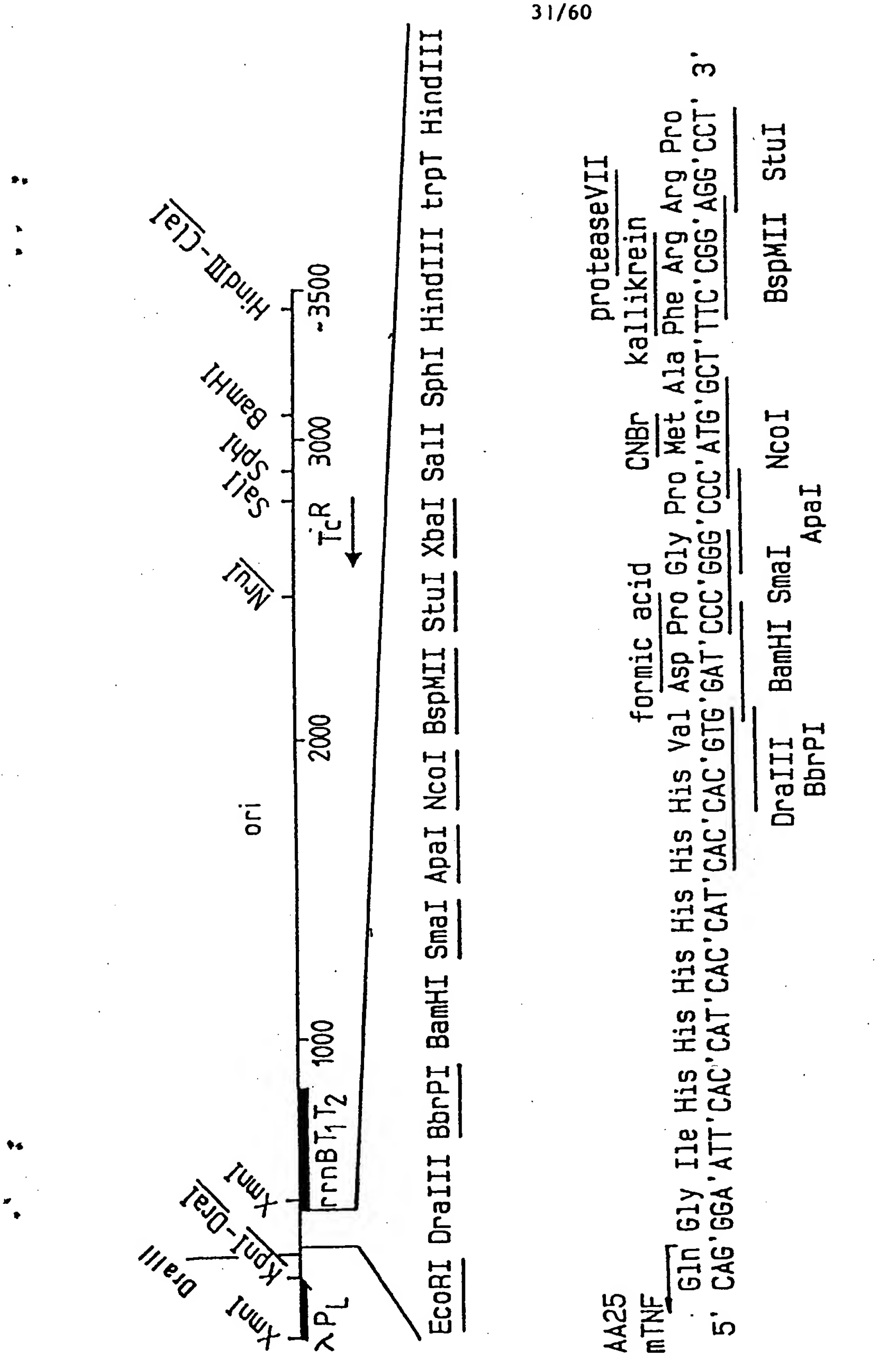
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DraIII
KpnI-DraI
XmnI
rrnBT1T2
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ori
2000
NruI
TcR
SalI
SphI
3000
BamHI
~3500
HindIII-ClaI
EcoRI DraIII BbrPI BamHI SmaI ApaI NcoI BspMII StuI XbaI SalI SphI HindIII trpT HindIII
AA25
mTNF
formic acid
CNBr
kallikrein
proteaseVII
Gln Gly Ile His His His His His His Val Asp Pro Gly Pro Met Ala Phe Arg Arg Pro
5' CAG'GGA'ATT'CAC'CAC'CAT'CAC'CAT'CAC'GTG'GAT'CCC'GGG'CCC'ATG'GCT'TTC'CGG'AGG'CCT' 3'
DraIII
BbrPI
BamHI SmaI
ApaI
NcoI
BspMII
StuI

fig.11a

From: pmTNF MPH

Fig. 11b

```
      3               9              15              21              27              33              39              45
      |               |               |               |               |               |               |               |
  1 AAT TCC GGG GAT CTC TCA CCT ACC AAA CAA TGC CCC CCT GCA AAA
    TTA AGG CCC CTA GAG AGT GGA TGG TTT GTT ACG GGG GGA CGT TTT

 46 AAT AAA TTC ATA TAA AAA ACA TAC AGA TAA CCA TCT GCG GTG ATA
    TTA TTT AAG TAT ATT TTT TGT ATG TCT ATT GGT AGA CGC CAC TAT

 91 AAT TAT CTC TGG CGG TGT TGA CAT AAA TAC CAC TGG CGG TGA TAC
    TTA ATA GAG ACC GCC ACA ACT GTA TTT ATG GTG ACC GCC ACT ATG

136 TGA GCA CAT CAG CAG GAC GCA CTG ACC ACC ATG AAG GTG ACG CTC
    ACT CGT GTA GTC GTC CTG CGT GAC TGG TGG TAC TTC CAC TGC GAG

181 TTA AAA ATT AAG CCC TGA AGA AGG GCA GGG GTA CCA GGA GGT TTA
    AAT TTT TAA TTC GGG ACT TCT TCC CGT CCC CAT GGT CCT CCA AAT

226 AAT CAT GGT AAG ATC AAG TAG TCA AAA TTC GAG TGA CAA GCC TGT
    TTA GTA CCA TTC TAG TTC ATC AGT TTT AAG CTC ACT GTT CGG ACA

271 AGC CCA CGT CGT AGC AAA CCA CCA AGT GGA GGA GCA GGG AAT TCA
    TCG GGT GCA GCA TCG TTT GGT GGT TCA CCT CCT CGT CCC TTA AGT

316 CCA TCA CCA TCA CCA CGT GGA TCC CGG GCC CAT GGC TTT CCG GAG
    GGT AGT GGT AGT GGT GCA CCT AGG GCC CGG GTA CCG AAA GGC CTC
```

Fig. 11b (Con't)

```
361 GCC TCT AGA GTC GAC CGG CAT GCA AGC TTA AGT AAG TAA GCC GCC
    CGG AGA TCT CAG CTG GCC GTA CGT TCG AAT TCA TTC ATT CGG CGG

406 AGT TCC GCT GGC GGC ATT TTN NTT GAT GCC CAA GCT TGG CTG TTT
    TCA AGG CGA CCG CCG TAA AAN NAA CTA CGG GTT CGA ACC GAC AAA

451 TGG CGG ATG AGA GAA GAT TTT CAG CCT GAT ACA GAT TAA ATC AGA
    ACC GCC TAC TCT CTT CTA AAA GTC GGA CTA TGT CTA ATT TAG TCT

496 ACG CAG AAG CGG TCT GAT AAA ACA GAA TTT GCC TGG CGG CAG TAG
    TGC GTC TTC GCC AGA CTA TTT TGT CTT AAA CGG ACC GCC GTC ATC

541 CGC GGT GGT CCC ACC TGA CCC CAT GCC GAA CTC AGA AGT GAA ACG
    GCG CCA CCA GGG TGG ACT GGG GTA CGG CTT GAG TCT TCA CTT TGC

586 CCG TAG CGC CGA TGG TAG TGT GGG GTC TCC CCA TGC GAG AGT AGG
    GGC ATC GCG GCT ACC ATC ACA CCC CAG AGG GGT ACG CTC TCA TCC

631 GAA CTG CCA GGC ATC AAA TAA AAC GAA AGG CTC AGT CGA AAG ACT
    CTT GAC GGT CCG TAG TTT ATT TTG CTT TCC GAG TCA GCT TTC TGA

676 GGG CCT TTC GTT TTA TCT GTT GTT TGT CGG TGA ACG CTC TCC TGA
    CCC GGA AAG CAA AAT AGA CAA CAA ACA GCC ACT TGC GAG AGG ACT

721 GTA GGA CAA ATC CGC CGG GAG CGG ATT TGA ACG TTG CGA AGC AAC
    CAT CCT GTT TAG GCG GCC CTC GCC TAA ACT TGC AAC GCT TCG TTG
```

Fig. 11b (Con't)

```
766  GGC CCG GAG GGT GGC GGG CAG GAC GCC CGC CAT AAA CTG CCA GGC
     CCG GGC CTC CCA CCG CCC GTC CTG CGG GCG GTA TTT GAC GGT CCG

811  ATC AAA TTA AGC AGA AGG CCA TCC TGA CGG ATG GCC TTT TTG CGT
     TAG TTT AAT TCG TCT TCC GGT AGG ACT GCC TAC CGG AAA AAC GCA

856  TTC TAC AAA CTC TTT TGT TTA TTT TTC TAA ATA CAT TCA AAT ATG
     AAG ATG TTT GAG AAA ACA AAT AAA AAG ATT TAT GTA AGT TTA TAC

901  TAT CCG CTC ATG AGA CAA TAA CCC TGA TAA ATG CTT CAA TAA TAA
     ATA GGC GAG TAC TCT GTT ATT GGG ACT ATT TAC GAA GTT ATT ATT

946  AAG GAT CTA GGT GAA GAT CCT TTT TGA TAA TCT CAT GAC CAA AAT
     TTC CTA GAT CCA CTT CTA GGA AAA ACT ATT AGA GTA CTG GTT TTA

991  CCC TTA ACG TGA GTT TTC GTT CCA CTG AGC GTC AGA CCC CGT AGA
     GGG AAT TGC ACT CAA AAG CAA GGT GAC TCG CAG TCT GGG GCA TCT

1036 AAA GAT CAA AGG ATC TTC TTG AGA TCC TTT TTT TCT GCG CGT AAT
     TTT CTA GTT TCC TAG AAG AAC TCT AGG AAA AAA AGA CGC GCA TTA
```

Fig. 11b (Con't)

1081 CTG CTG CTT GCA AAC AAA AAA ACC ACC GCT ACC AGC GGT GGT TTG
GAC GAC GAA CGT TTG TTT TTT TGG TGG CGA TGG TCG CCA CCA AAC

1126 TTT GCC GGA TCA AGA GCT ACC AAC TCT TTT TCC GAA GGT AAC TGG
AAA CGG CCT AGT TCT CGA TGG TTG AGA AAA AGG CTT CCA TTG ACC

1171 CTT CAG CAG AGC GCA GAT ACC AAA TAC TGT CCT TCT AGT GTA GCC
GAA GTC GTC TCG CGT CTA TGG TTT ATG ACA GGA AGA TCA CAT CGG

1216 GTA GTT AGG CCA CCA CTT CAA GAA CTC TGT AGC ACC GCC TAC ATA
CAT CAA TCC GGT GGT GAA GTT CTT GAG ACA TCG TGG CGG ATG TAT

1261 CCT CGC TCT GCT AAT CCT GTT ACC AGT GGC TGC TGC CAG TGG CGA
GGA GCG AGA CGA TTA GGA CAA TGG TCA CCG ACG ACG GTC ACC GCT

1306 TAA GTC GTG TCT TAC CGG GTT GGA CTC AAG ACG ATA GTT ACC GGA
ATT CAG CAC AGA ATG GCC CAA CCT GAG TTC TGC TAT CAA TGG CCT

1351 TAA GGC GCA GCG GTC GGG CTG AAC GGG GGG TTC GTG CAC ACA GCC
ATT CCG CGT CGC CAG CCC GAC TTG CCC CCC AAG CAC GTG TGT CGG

Fig. 11b (Con't)

```
1396 CAG CTT GGA GCG AAC GAC CTA CAC CGA ACT GAG ATA CCT ACA GCG
     GTC GAA CCT CGC TTG CTG GAT GTG GCT TGA CTC TAT GGA TGT CGC

1441 TGA GCA TTG AGA AAG CGC CAC GCT TCC CGA AGG GAG AAA GGC GGA
     ACT CGT AAC TCT TTC GCG GTG CGA AGG GCT TCC CTC TTT CCG CCT

1486 CAG GTA TCC GGT AAG CGG CAG GGT CGG AAC AGG AGA GCG CAC GAG
     GTC CAT AGG CCA TTC GCC GTC CCA GCC TTG TCC TCT CGC GTG CTC

1531 GGA GCT TCC AGG GGG AAA CGC CTG GTA TCT TTA TAG TCC TGT CGG
     CCT CGA AGG TCC CCC TTT GCG GAC CAT AGA AAT ATC AGG ACA GCC

1576 GTT TCG CCA CCT CTG ACT TGA GCG TCG ATT TTT GTG ATG CTC GTC
     CAA AGC GGT GGA GAC TGA ACT CGC AGC TAA AAA CAC TAC GAG CAG
```

Fig. 11b (Con't)

```
1621 AGG GGG GCG GAG CCT ATG GAA AAA CGC CAG CAA CGC GGC CTT TTT
     TCC CCC CGC CTC GGA TAC CTT TTT GCG GTC GTT GCG CCG GAA AAA

1666 ACG GTT CCT GGC CTT TTG CTG GCC TTT TGC TCA CAT GTT CTT TCC
     TGC CAA GGA CCG GAA AAC GAC CGG AAA ACG AGT GTA CAA GAA AGG

1711 TGC GTT ATC CCC TGA TTC TGT GGA TAA CCG TAT TAC CGC CTT TGA
     ACG CAA TAG GGG ACT AAG ACA CCT ATT GGC ATA ATG GCG GAA ACT

1756 GTG AGC TGA TAC CGC TCG CCG CAG CCG AAC GAC CGA GCG CAG CGA
     CAC TCG ACT ATG GCG AGC GGC GTC GGC TTG CTG GCT CGC GTC GCT

1801 GTC AGT GAG CGA GGA AGC GGA AGA GCG CTG ACT TCC GCG TTT CCA
     CAG TCA CTC GCT CCT TCG CCT TCT CGC GAC TGA AGG CGC AAA GGT

1846 GAC TTT ACG AAA CAC GGA AAC CGA AGA CCA TTC ATG TTG TTG CTC
     CTG AAA TGC TTT GTG CCT TTG GCT TCT GGT AAG TAC AAC AAC GAG

1891 AGG TCG CAG ACG TTT TGC AGC AGC AGT CGC TTC ACG TTC GCT CGC
     TCC AGC GTC TGC AAA ACG TCG TCG TCA GCG AAG TGC AAG CGA GCG
```

Fig. 11b (Con't)

1936 GTA TCG GTG ATT CAT TCT GCT AAC CAG TAA GGC AAC CCC GCC AGC
CAT AGC CAC TAA GTA AGA CGA TTG GTC ATT CCG TTG GGG CGG TCG

1981 CTA GCC GGG TCC TCA ACG ACA GGA GCA CGA TCA TGC GCA CCC GTG
GAT CGG CCC AGG AGT TGC TGT CCT CGT GCT AGT ACG CGT GGG CAC

2026 GCC AGG ACC CAA CGC TGC CCG AGA TGC GCC GCG TGC GGC TGC TGG
CGG TCC TGG GTT GCG ACG GGC TCT ACG CGG CGC ACG CCG ACG ACC

2071 AGA TGG CGG ACG CGA TGG ATA TGT TCT GCC AAG GGT TGG TTT GCG
TCT ACC GCC TGC GCT ACC TAT ACA AGA CGG TTC CCA ACC AAA CGC

2116 CAT TCA CAG TTC TCC GCA AGA ATT GAT TGG CTC CAA TTC TTG GAG
GTA AGT GTC AAG AGG CGT TCT TAA CTA ACC GAG GTT AAG AAC CTC

2161 TGG TGA ATC CGT TAG CGA GGT GCC GCC GGC TTC CAT TCA GGT CGA
ACC ACT TAG GCA ATC GCT CCA CGG CGG CCG AAG GTA AGT CCA GCT

2206 GGT GGC CCG GCT CCA TGC ACC GCG ACG CAA CGC GGG GAG GCA GAC
CCA CCG GGC CGA GGT ACG TGG CGC TGC GTT GCG CCC CTC CGT CTG

Fig. 11b (Con't)

```
2251 AAG GTA TAG GGC GGC GCC TAC AAT CCA TGC CAA CCC GTT CCA TGT
     TTC CAT ATC CCG CCG CGG ATG TTA GGT ACG GTT GGG CAA GGT ACA

2296 GCT CGC CGA GGC GGC ATA AAT CGC CGT GAC GAT CAG CGG TCC AGT
     CGA GCG GCT CCG CCG TAT TTA GCG GCA CTG CTA GTC GCC AGG TCA

2341 GAT CGA AGT TAG GCT GGT AAG AGC CGC GAG CGA TCC TTG AAG CTG
     CTA GCT TCA ATC CGA CCA TTC TCG GCG CTC GCT AGG AAC TTC GAC

2386 TCC CTG ATG GTC GTC ATC TAC CTG CCT GGA CAG CAT GGC CTG CAA
     AGG GAC TAC CAG CAG TAG ATG GAC GGA CCT GTC GTA CCG GAC GTT

2431 CGC GGG CAT CCC GAT GCC GCC GGA AGC GAG AAG AAT CAT AAT GGG
     GCG CCC GTA GGG CTA CGG CGG CCT TCG CTC TTC TTA GTA TTA CCC
```

Fig. 11b (Con't)

```
2476 GAA GGC CAT CCA GCC TCG CGT CGC GAA CGC CAG CAA GAC GTA GCC
     CTT CCG GTA GGT CGG AGC GCA GCG CTT GCG GTC GTT CTG CAT CGG

2521 CAG CGC GTC GGC CGC CAT GCC GGC GAT AAT GGC CTG CTT CTC GCC
     GTC GCG CAG CCG GCG GTA CGG CCG CTA TTA CCG GAC GAA GAG CGG

2566 GAA ACG TTT GGT GGC GGG ACC AGT GAC GAA GGC TTG AGC GAG GGC
     CTT TGC AAA CCA CCG CCC TGG TCA CTG CTT CCG AAC TCG CTC CCG

2611 GTG CAA GAT TCC GAA TAC CGC AAG CGA CAG GCC GAT CAT CGT CGC
     CAC GTT CTA AGG CTT ATG GCG TTC GCT GTC CGG CTA GTA GCA GCG

2656 GCT CCA GCG AAA GCG GTC CTC GCC GAA AAT GAC CCA GAG CGC TGC
     CGA GGT CGC TTT CGC CAG GAG CGG CTT TTA CTG GGT CTC GCG ACG

2701 CGG CAC CTG TCC TAC GAG TTG CAT GAT AAA GAA GAC AGT CAT AAG
     GCC GTG GAC AGG ATG CTC AAC GTA CTA TTT CTT CTG TCA GTA TTC

2746 TGC GGC GAC GAT AGT CAT GCC CCG CGC CCA CCG GAA GGA GCT GAC
     ACG CCG CTG CTA TCA GTA CGG GGC GCG GGT GGC CTT CCT CGA CTG
```

Fig. 11b (Con't)

```
2791 TGG GTT GAA GGC TCT CAA GGG CAT CGG TCG ACG CTC TCC CTT ATG
     ACC CAA CTT CCG AGA GTT CCC GTA GCC AGC TGC GAG AGG GAA TAC

2836 CGA CTC CTG CAT TAG GAA GCA GCC CAG TAG TAG GTT GAG GCC GTT
     GCT GAG GAC GTA ATC CTT CGT CGG GTC ATC ATC CAA CTC CGG CAA

2881 GAG CAC CGC CGC CGC AAG GAA TGG TGC ATG CAA GGA GAT GGC GCC
     CTC GTG GCG GCG GCG TTC CTT ACC ACG TAC GTT CCT CTA CCG CGG

2926 CAA CAG TCC CCC GGC CAC GGG GCC TGC CAC CAT ACC CAC GCC GAA
     GTT GTC AGG GGG CCG GTG CCC CGG ACG GTG GTA TGG GTG CGG CTT

2971 ACA AGC GCT CAT GAG CCC GAA GTG GCG AGC CCG ATC TTC CCC ATC
     TGT TCG CGA GTA CTC GGG CTT CAC CGC TCG GGC TAG AAG GGG TAG

3016 GGT GAT GTC GGC GAT ATA GGC GCC AGC AAC CGC ACC TGT GGC GCC
     CCA CTA CAG CCG CTA TAT CCG CGG TCG TTG GCG TGG ACA CCG CGG

3061 GGT GAT GCC GGC CAC GAT GCG TCC GGC GTA GAG GAT CCA CAG GAC
     CCA CTA CGG CCG GTG CTA CGC AGG CCG CAT CTC CTA GGT GTC CTG
```

Fig. 11b (Con't)

```
3106 GGG TGT GGT CGC CAT GAT CGC GTA GTC GAT AGT GGC TCC AAG TAG
     CCC ACA CCA GCG GTA CTA GCG CAT CAG CTA TCA CCG AGG TTC ATC

3151 CGA AGC GAG CAG GAC TGG GCG GCG GCC AAA GCG GTC GGA CAG TGC
     GCT TCG CTC GTC CTG ACC CGC CGC CGG TTT CGC CAG CCT GTC ACG

3196 TCC GAG AAC GGG TGC GCA TAG AAA TTG CAT CAA CGC ATA TAG CGC
     AGG CTC TTG CCC ACG CGT ATC TTT AAC GTA GTT GCG TAT ATC GCG

3241 TAG CAG CAC GCC ATA GTG ACT GGC GAT GCT GTC GGA ATG GAC GAT
     ATC GTC GTG CGG TAT CAC TGA CCG CTA CGA CAG CCT TAC CTG CTA

3286 ATC CCG CAA GAG GCC CGG CAG TAC CGG CAT AAC CAA GCC TAT GCC
     TAG GGC GTT CTC CGG GCC GTC ATG GCC GTA TTG GTT CGG ATA CGG
```

Fig. 11b (Con't)

```
3331 TAC AGC ATC CAG GGT GAC GGT GCC GAG GAT GAC GAT GAG CGC ATT
     ATG TCG TAG GTC CCA CTG CCA CGG CTC CTA CTG CTA CTC GCG TAA

3376 GTT AGA TTT CAT ACA CGG TGC CTG ACT GCG TTA GCA ATT TAA CTG
     CAA TCT AAA GTA TGT GCC ACG GAC TGA CGC AAT CGT TAA ATT GAC

3421 TGA TAA ACT ACC GCA TTA AAG CTT ATC GAT GAT AAG CTG TCA AAC
     ACT ATT TGA TGG CGT AAT TTC GAA TAG CTA CTA TTC GAC AGT TTG

3466 ATG AGA ATT
     TAC TCT TAA
```

Total number of bases is: 3474.
DNA sequence composition: 845 A; 933 C; 978 G; 716 T;
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Sequence name: NPMTNFMPH.

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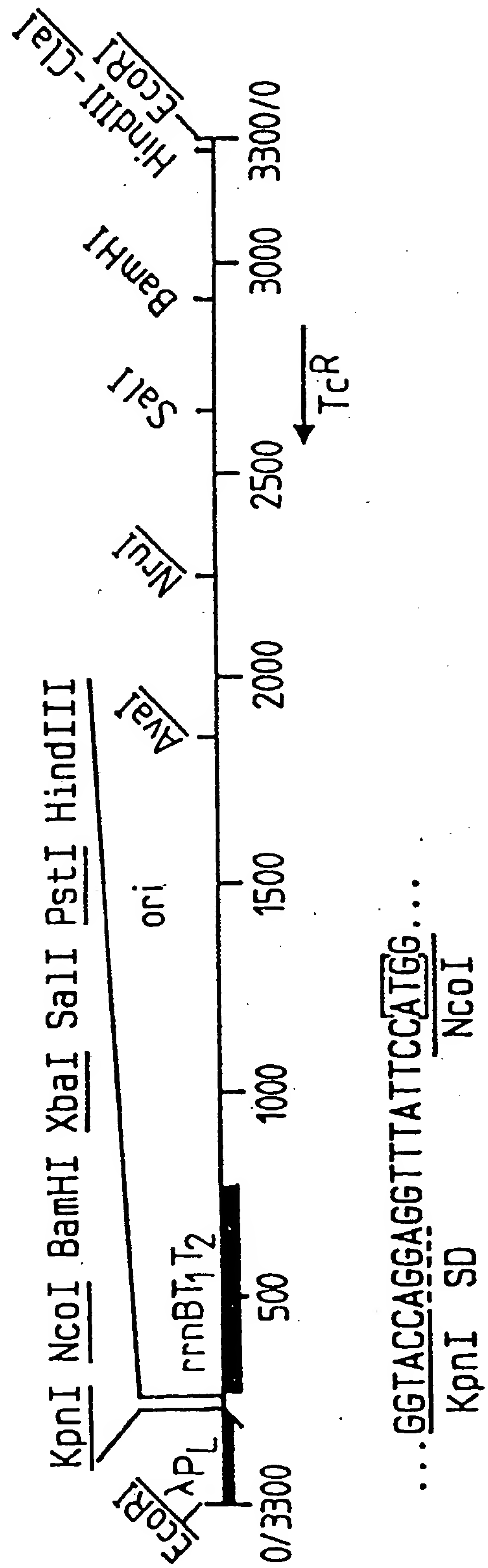
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2500
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TcR
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KpnI
SD
NcoI

fig.12a

From: pIG2

Fig. 12b

```
           3           9          15          21          27          33          39          45
           |           |           |           |           |           |           |           |
  1  TTC CGG GGA TCT CTC ACC TAC CAA ACA ATG CCC CCC TGC AAA AAA
     AAG GCC CCT AGA GAG TGG ATG GTT TGT TAC GGG GGG ACG TTT TTT

 46  TAA ATT CAT ATA AAA AAC ATA CAG ATA ACC ATC TGC GGT GAT AAA
     ATT TAA GTA TAT TTT TTG TAT GTC TAT TGG TAG ACG CCA CTA TTT

 91  TTA TCT CTG GCG GTG TTG ACA TAA ATA CCA CTG GCG GTG ATA CTG
     AAT AGA GAC CGC CAC AAC TGT ATT TAT GGT GAC CGC CAC TAT GAC

136  AGC ACA TCA GCA GGA CGC ACT GAC CAC CAT GAA GGT GAC GCT CTT
     TCG TGT AGT CGT CCT GCG TGA CTG GTG GTA CTT CCA CTG CGA GAA

181  AAA AAT TAA GCC CTG AAG AAG GGC AGG GGT ACC AGG AGG TTT AAA
     TTT TTA ATT CGG GAC TTC TTC CCG TCC CCA TGG TCC TCC AAA TTT

226  TAT TCC ATG GGG GGG ATC CTC TAG AGT CGA CCT GCA GCC CAA GCT
     ATA AGG TAC CCC CCC TAG GAG ATC TCA GCT GGA CGT CGG GTT CGA

271  TGG CTG TTT TGG CGG ATG AGA GAA GAT TTT CAG CCT GAT ACA GAT
     ACC GAC AAA ACC GCC TAC TCT CTT CTA AAA GTC GGA CTA TGT CTA

316  TAA ATC AGA ACG CAG AAG CGG TCT GAT AAA ACA GAA TTT GCC TGG
     ATT TAG TCT TGC GTC TTC GCC AGA CTA TTT TGT CTT AAA CGG ACC
```

Fig. 12b

```
361 CGG CAG TAG CGC GGT GGT CCC ACC TGA CCC CAT GCC GAA CTC AGA
    GCC GTC ATC GCG CCA CCA GGG TGG ACT GGG GTA CGG CTT GAG TCT
406 AGT GAA ACG CCG TAG CGC CGA TGG TAG TGT GGG GTC TCC CCA TGC
    TCA CTT TGC GGC ATC GCG GCT ACC ATC ACA CCC CAG AGG GGT ACG

451 GAG AGT AGG GAA CTG CCA GGC ATC AAA TAA AAC GAA AGG CTC AGT
    CTC TCA TCC CTT GAC GGT CCG TAG TTT ATT TTG CTT TCC GAG TCA

496 CGA AAG ACT GGG CCT TTC GTT TTA TCT GTT GTT TGT CGG TGA ACG
    GCT TTC TGA CCC GGA AAG CAA AAT AGA CAA CAA ACA GCC ACT TGC

541 CTC TCC TGA GTA GGA CAA ATC CGC CGG GAG CGG ATT TGA ACG TTG
    GAG AGG ACT CAT CCT GTT TAG GCG GCC CTC GCC TAA ACT TGC AAC

586 CGA AGC AAC GGC CCG GAG GGT GGC GGG CAG GAC GCC CGC CAT AAA
    GCT TCG TTG CCG GGC CTC CCA CCG CCC GTC CTG CGG GCG GTA TTT

631 CTG CCA GGC ATC AAA TTA AGC AGA AGG CCA TCC TGA CGG ATG GCC
    GAC GGT CCG TAG TTT AAT TCG TCT TCC GGT AGG ACT GCC TAC CGG

676 TTT TTG CGT TTC TAC AAA CTC TTT TGT TTA TTT TTC TAA ATA CAT
    AAA AAC GCA AAG ATG TTT GAG AAA ACA AAT AAA AAG ATT TAT GTA

721 TCA AAT ATG TAT CCG CTC ATG AGA CAA TAA CCC TGA TAA ATG CTT
    AGT TTA TAC ATA GGC GAG TAC TCT GTT ATT GGG ACT ATT TAC GAA
```

Fig. 12b (Con't)

```
766  CAA TAA TAA AAG GAT CTA GGT GAA GAT CCT TTT TGA TAA TCT CAT
     GTT ATT ATT TTC CTA GAT CCA CTT CTA GGA AAA ACT ATT AGA GTA

811  GAC CAA AAT CCC TTA ACG TGA GTT TTC GTT CCA CTG AGC GTC AGA
     CTG GTT TTA GGG AAT TGC ACT CAA AAG CAA GGT GAC TCG CAG TCT

856  CCC CGT AGA AAA GAT CAA AGG ATC TTC TTG AGA TCC TTT TTT TCT
     GGG GCA TCT TTT CTA GTT TCC TAG AAG AAC TCT AGG AAA AAA AGA

901  GCG CGT AAT CTG CTG CTT GCA AAC AAA AAA ACC ACC GCT ACC AGC
     CGC GCA TTA GAC GAC GAA CGT TTG TTT TTT TGG TGG CGA TGG TCG

946  GGT GGT TTG TTT GCC GGA TCA AGA GCT ACC AAC TCT TTT TCC GAA
     CCA CCA AAC AAA CGG CCT AGT TCT CGA TGG TTG AGA AAA AGG CTT

991  GGT AAC TGG CTT CAG CAG AGC GCA GAT ACC AAA TAC TGT CCT TCT
     CCA TTG ACC GAA GTC GTC TCG CGT CTA TGG TTT ATG ACA GGA AGA

1036 AGT GTA GCC GTA GTT AGG CCA CCA CTT CAA GAA CTC TGT AGC ACC
     TCA CAT CGG CAT CAA TCC GGT GGT GAA GTT CTT GAG ACA TCG TGG
```

Fig. 12b (Con't)

```
1081 GCC TAC ATA CCT CGC TCT GCT AAT CCT GTT ACC AGT GGC TGC TGC
     CGG ATG TAT GGA GCG AGA CGA TTA GGA CAA TGG TCA CCG ACG ACG

1126 CAG TGG CGA TAA GTC GTG TCT TAC CGG GTT GGA CTC AAG ACG ATA
     GTC ACC GCT ATT CAG CAC AGA ATG GCC CAA CCT GAG TTC TGC TAT

1171 GTT ACC GGA TAA GGC GCA GCG GTC GGG CTG AAC GGG GGG TTC GTG
     CAA TGG CCT ATT CCG CGT CGC CAG CCC GAC TTG CCC CCC AAG CAC

1216 CAC ACA GCC CAG CTT GGA GCG AAC GAC CTA CAC CGA ACT GAG ATA
     GTG TGT CGG GTC GAA CCT CGC TTG CTG GAT GTG GCT TGA CTC TAT

1261 CCT ACA GCG TGA GCA TTG AGA AAG CGC CAC GCT TCC CGA AGG GAG
     GGA TGT CGC ACT CGT AAC TCT TTC GCG GTG CGA AGG GCT TCC CTC

1306 AAA GGC GGA CAG GTA TCC GGT AAG CGG CAG GGT CGG AAC AGG AGA
     TTT CCG CCT GTC CAT AGG CCA TTC GCC GTC CCA GCC TTG TCC TCT

1351 GCG CAC GAG GGA GCT TCC AGG GGG AAA CGC CTG GTA TCT TTA TAG
     CGC GTG CTC CCT CGA AGG TCC CCC TTT GCG GAC CAT AGA AAT ATC
```

Fig. 12b (Con't)

```
1396 TCC TGT CGG GTT TCG CCA CCT CTG ACT TGA GCG TCG ATT TTT GTG
     AGG ACA GCC CAA AGC GGT GGA GAC TGA ACT CGC AGC TAA AAA CAC

1441 ATG CTC GTC AGG GGG GCG GAG CCT ATG GAA AAA CGC CAG CAA CGC
     TAC GAG CAG TCC CCC CGC CTC GGA TAC CTT TTT GCG GTC GTT GCG

1486 GGC CTT TTT ACG GTT CCT GGC CTT TTG CTG GCC TTT TGC TCA CAT
     CCG GAA AAA TGC CAA GGA CCG GAA AAC GAC CGG AAA ACG AGT GTA

1531 GTT CTT TCC TGC GTT ATC CCC TGA TTC TGT GGA TAA CCG TAT TAC
     CAA GAA AGG ACG CAA TAG GGG ACT AAG ACA CCT ATT GGC ATA ATG

1576 CGC CTT TGA GTG AGC TGA TAC CGC TCG CCG CAG CCG AAC GAC CGA
     GCG GAA ACT CAC TCG ACT ATG GCG AGC GGC GTC GGC TTG CTG GCT
```

Fig. 12b (Con't)

```
1621 GCG CAG CGA GTC AGT GAG CGA GGA AGC GGA AGA GCG CTG ACT TCC
     CGC GTC GCT CAG TCA CTC GCT CCT TCG CCT TCT CGC GAC TGA AGG

1666 GCG TTT CCA GAC TTT ACG AAA CAC GGA AAC CGA AGA CCA TTC ATG
     CGC AAA GGT CTG AAA TGC TTT GTG CCT TTG GCT TCT GGT AAG TAC

1711 TTG TTG CTC AGG TCG CAG ACG TTT TGC AGC AGC AGT CGC TTC ACG
     AAC AAC GAG TCC AGC GTC TGC AAA ACG TCG TCG TCA GCG AAG TGC

1756 TTC GCT CGC GTA TCG GTG ATT CAT TCT GCT AAC CAG TAA GGC AAC
     AAG CGA GCG CAT AGC CAC TAA GTA AGA CGA TTG GTC ATT CCG TTG

1801 CCC GCC AGC CTA GCC GGG TCC TCA ACG ACA GGA GCA CGA TCA TGC
     GGG CGG TCG GAT CGG CCC AGG AGT TGC TGT CCT CGT GCT AGT ACG

1846 GCA CCC GTG GCC AGG ACC CAA CGC TGC CCG AGA TGC GCC GCG TGC
     CGT GGG CAC CGG TCC TGG GTT GCG ACG GGC TCT ACG CGG CGC ACG

1891 GGC TGC TGG AGA TGG CGG ACG CGA TGG ATA TGT TCT GCC AAG GGT
     CCG ACG ACC TCT ACC GCC TGC GCT ACC TAT ACA AGA CGG TTC CCA
```

Fig. 12b (Con't)

```
1936 TGG TTT GCG CAT TCA CAG TTC TCC GCA AGA ATT GAT TGG CTC CAA
     ACC AAA CGC GTA AGT GTC AAG AGG CGT TCT TAA CTA ACC GAG GTT

1981 TTC TTG GAG TGG TGA ATC CGT TAG CGA GGT GCC GCC GGC TTC CAT
     AAG AAC CTC ACC ACT TAG GCA ATC GCT CCA CGG CGG CCG AAG GTA

2026 TCA GGT CGA GGT GGC CCG GCT CCA TGC ACC GCG ACG CAA CGC GGG
     AGT CCA GCT CCA CCG GGC CGA GGT ACG TGG CGC TGC GTT GCG CCC

2071 GAG GCA GAC AAG GTA TAG GGC GGC GCC TAC AAT CCA TGC CAA CCC
     CTC CGT CTG TTC CAT ATC CCG CCG CGG ATG TTA GGT ACG GTT GGG

2116 GTT CCA TGT GCT CGC CGA GGC GGC ATA AAT CGC CGT GAC GAT CAG
     CAA GGT ACA CGA GCG GCT CCG CCG TAT TTA GCG GCA CTG CTA GTC

2161 CGG TCC AGT GAT CGA AGT TAG GCT GGT AAG AGC CGC GAG CGA TCC
     GCC AGG TCA CTA GCT TCA ATC CGA CCA TTC TCG GCG CTC GCT AGG

2206 TTG AAG CTG TCC CTG ATG GTC GTC ATC TAC CTG CCT GGA CAG CAT
     AAC TTC GAC AGG GAC TAC CAG CAG TAG ATG GAC GGA CCT GTC GTA
```

Fig. 12b (Con't)

```
2251 GGC CTG CAA CGC GGG CAT CCC GAT GCC GCC GGA AGC GAG AAG AAT
     CCG GAC GTT GCG CCC GTA GGG CTA CGG CGG CCT TCG CTC TTC TTA

2296 CAT AAT GGG GAA GGC CAT CCA GCC TCG CGT CGC GAA CGC CAG CAA
     GTA TTA CCC CTT CCG GTA GGT CGG AGC GCA GCG CTT GCG GTC GTT

2341 GAC GTA GCC CAG CGC GTC GGC CGC CAT GCC GGC GAT AAT GGC CTG
     CTG CAT CGG GTC GCG CAG CCG GCG GTA CGG CCG CTA TTA CCG GAC

2386 CTT CTC GCC GAA ACG TTT GGT GGC GGG ACC AGT GAC GAA GGC TTG
     GAA GAG CGG CTT TGC AAA CCA CCG CCC TGG TCA CTG CTT CCG AAC

2431 AGC GAG GGC GTG CAA GAT TCC GAA TAC CGC AAG CGA CAG GCC GAT
     TCG CTC CCG CAC GTT CTA AGG CTT ATG GCG TTC GCT GTC CGG CTA
```

Fig. 12b (Con't)

```
2476 CAT CGT CGC GCT CCA GCG AAA GCG GTC CTC GCC GAA AAT GAC CCA
     GTA GCA GCG CGA GGT CGC TTT CGC CAG GAG CGG CTT TTA CTG GGT

2521 GAG CGC TGC CGG CAC CTG TCC TAC GAG TTG CAT GAT AAA GAA GAC
     CTC GCG ACG GCC GTG GAC AGG ATG CTC AAC GTA CTA TTT CTT CTG

2566 AGT CAT AAG TGC GGC GAC GAT AGT CAT GCC CCG CGC CCA CCG GAA
     TCA GTA TTC ACG CCG CTG CTA TCA GTA CGG GGC GCG GGT GGC CTT

2611 GGA GCT GAC TGG GTT GAA GGC TCT CAA GGG CAT CGG TCG ACG CTC
     CCT CGA CTG ACC CAA CTT CCG AGA GTT CCC GTA GCC AGC TGC GAG

2656 TCC CTT ATG CGA CTC CTG CAT TAG GAA GCA GCC CAG TAG TAG GTT
     AGG GAA TAC GCT GAG GAC GTA ATC CTT CGT CGG GTC ATC ATC CAA

2701 GAG GCC GTT GAG CAC CGC CGC CGC AAG GAA TGG TGC ATG CAA GGA
     CTC CGG CAA CTC GTG GCG GCG GCG TTC CTT ACC ACG TAC GTT CCT

2746 GAT GGC GCC CAA CAG TCC CCC GGC CAC GGG GCC TGC CAC CAT ACC
     CTA CCG CGG GTT GTC AGG GGG CCG GTG CCC CGG ACG GTG GTA TGG
```

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Fig. 12b (Con't)

```
2791 CAC GCC GAA ACA AGC GCT CAT GAG CCC GAA GTG GCG AGC CCG ATC
     GTG CGG CTT TGT TCG CGA GTA CTC GGG CTT CAC CGC TCG GGC TAG

2836 TTC CCC ATC GGT GAT GTC GGC GAT ATA GGC GCC AGC AAC CGC ACC
     AAG GGG TAG CCA CTA CAG CCG CTA TAT CCG CGG TCG TTG GCG TGG

2881 TGT GGC GCC GGT GAT GCC GGC CAC GAT GCG TCC GGC GTA GAG GAT
     ACA CCG CGG CCA CTA CGG CCG GTG CTA CGC AGG CCG CAT CTC CTA

2926 CCA CAG GAC GGG TGT GGT CGC CAT GAT CGC GTA GTC GAT AGT GGC
     GGT GTC CTG CCC ACA CCA GCG GTA CTA GCG CAT CAG CTA TCA CCG

2971 TCC AAG TAG CGA AGC GAG CAG GAC TGG GCG GCG GCC AAA GCG GTC
     AGG TTC ATC GCT TCG CTC GTC CTG ACC CGC CGC CGG TTT CGC CAG

3016 GGA CAG TGC TCC GAG AAC GGG TGC GCA TAG AAA TTG CAT CAA CGC
     CCT GTC ACG AGG CTC TTG CCC ACG CGT ATC TTT AAC GTA GTT GCG

3061 ATA TAG CGC TAG CAG CAC GCC ATA GTG ACT GGC GAT GCT GTC GGA
     TAT ATC GCG ATC GTC GTG CGG TAT CAC TGA CCG CTA CGA CAG CCT
```

Fig. 12b (Con't)

```
3106 ATG GAC GAT ATC CCG CAA GAG GCC CGG CAG TAC CGG CAT AAC CAA
     TAC CTG CTA TAG GGC GTT CTC CGG GCC GTC ATG GCC GTA TTG GTT

3151 GCC TAT GCC TAC AGC ATC CAG GGT GAC GGT GCC GAG GAT GAC GAT
     CGG ATA CGG ATG TCG TAG GTC CCA CTG CCA CGG CTC CTA CTG CTA

3196 GAG CGC ATT GTT AGA TTT CAT ACA CGG TGC CTG ACT GCG TTA GCA
     CTC GCG TAA CAA TCT AAA GTA TGT GCC ACG GAC TGA CGC AAT CGT

3241 ATT TAA CTG TGA TAA ACT ACC GCA TTA AAG CTT ATC GAT GAT AAG
     TAA ATT GAC ACT ATT TGA TGG CGT AAT TTC GAA TAG CTA CTA TTC

3286 CTG TCA AAC ATG AGA A
     GAC AGT TTG TAC TCT T
```

Total number of bases is: 3301.
DNA sequence composition: 797 A; 887 C; 936 G; 681 T;

Sequence name: NIPS0039.

==

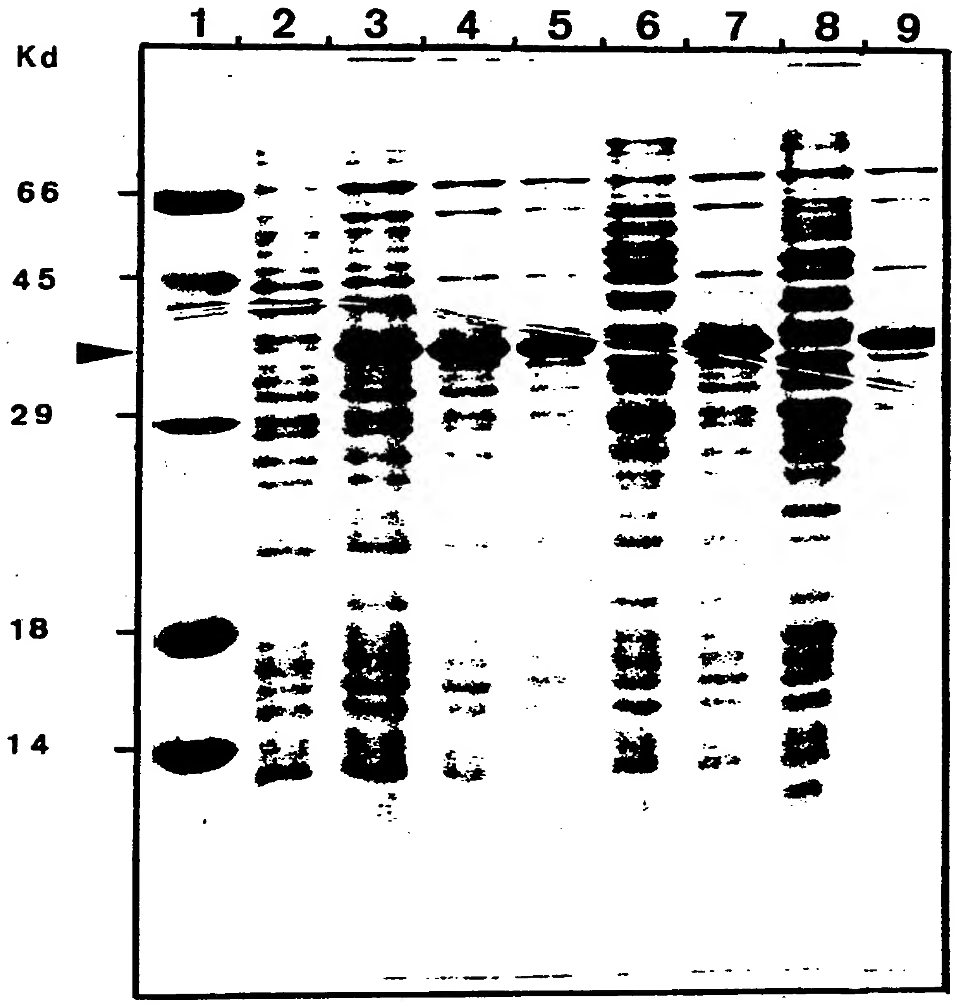


fig. 14a

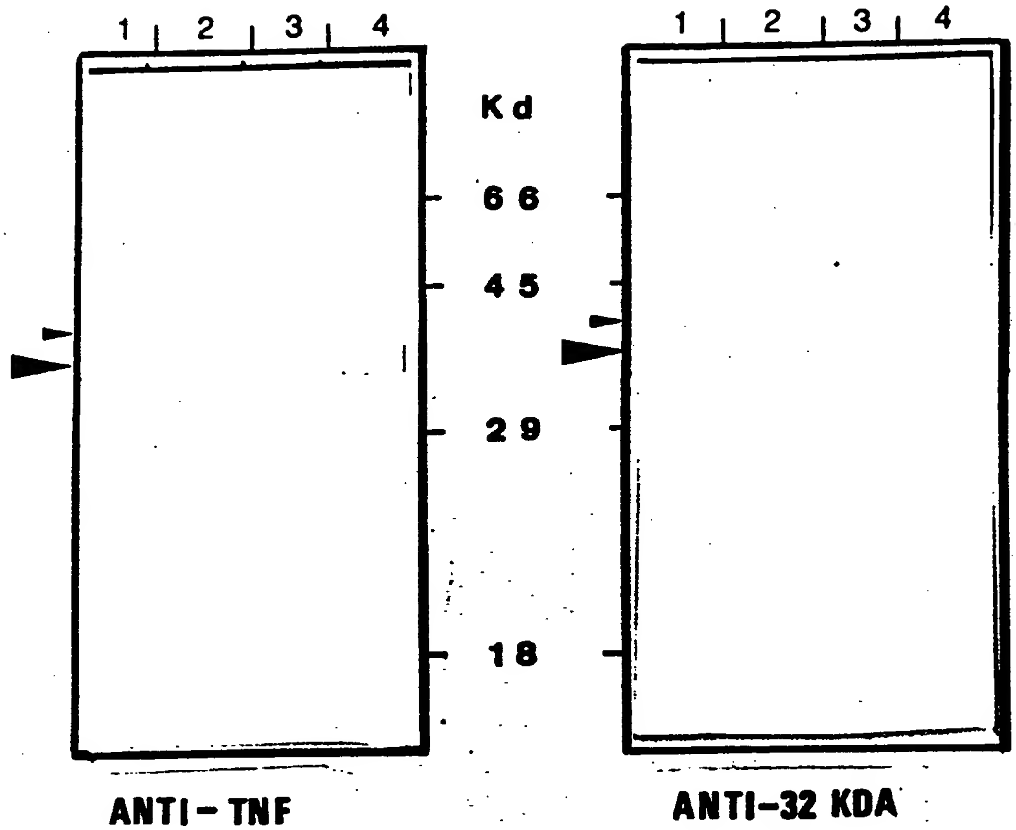
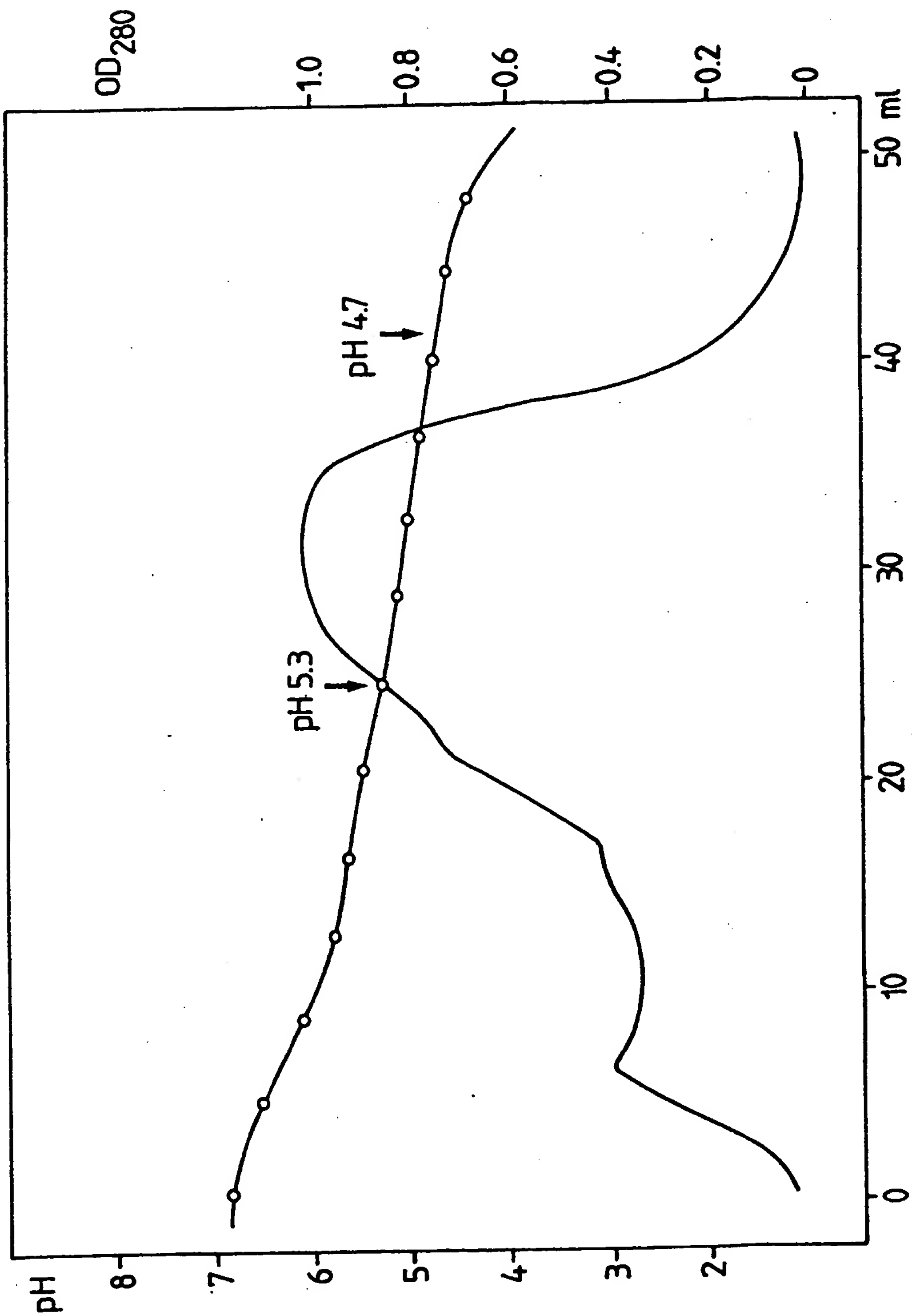
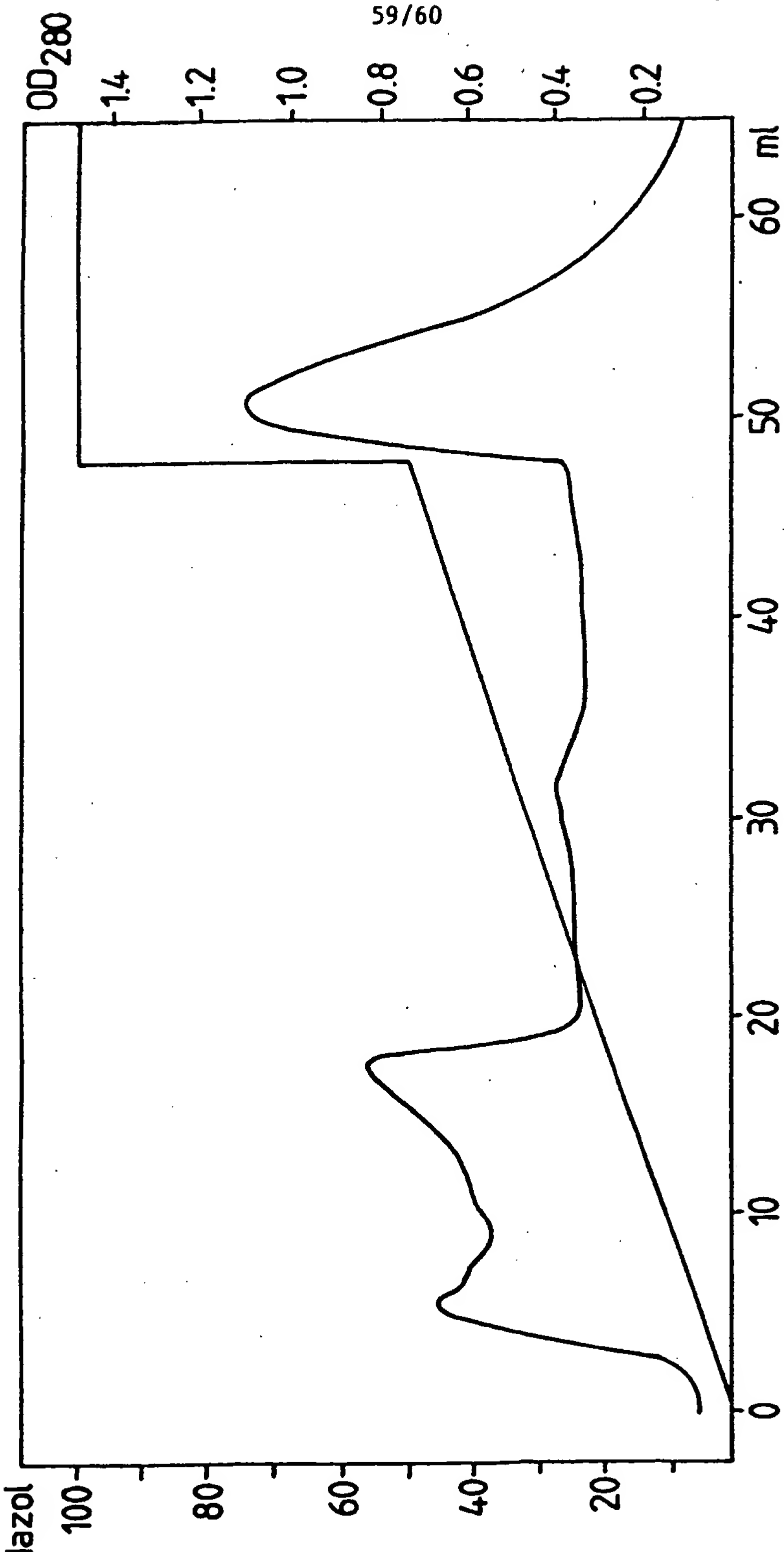


fig.14b

FIG. 15

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1.2
1.0
0.8
0.6
0.4
0.2
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30
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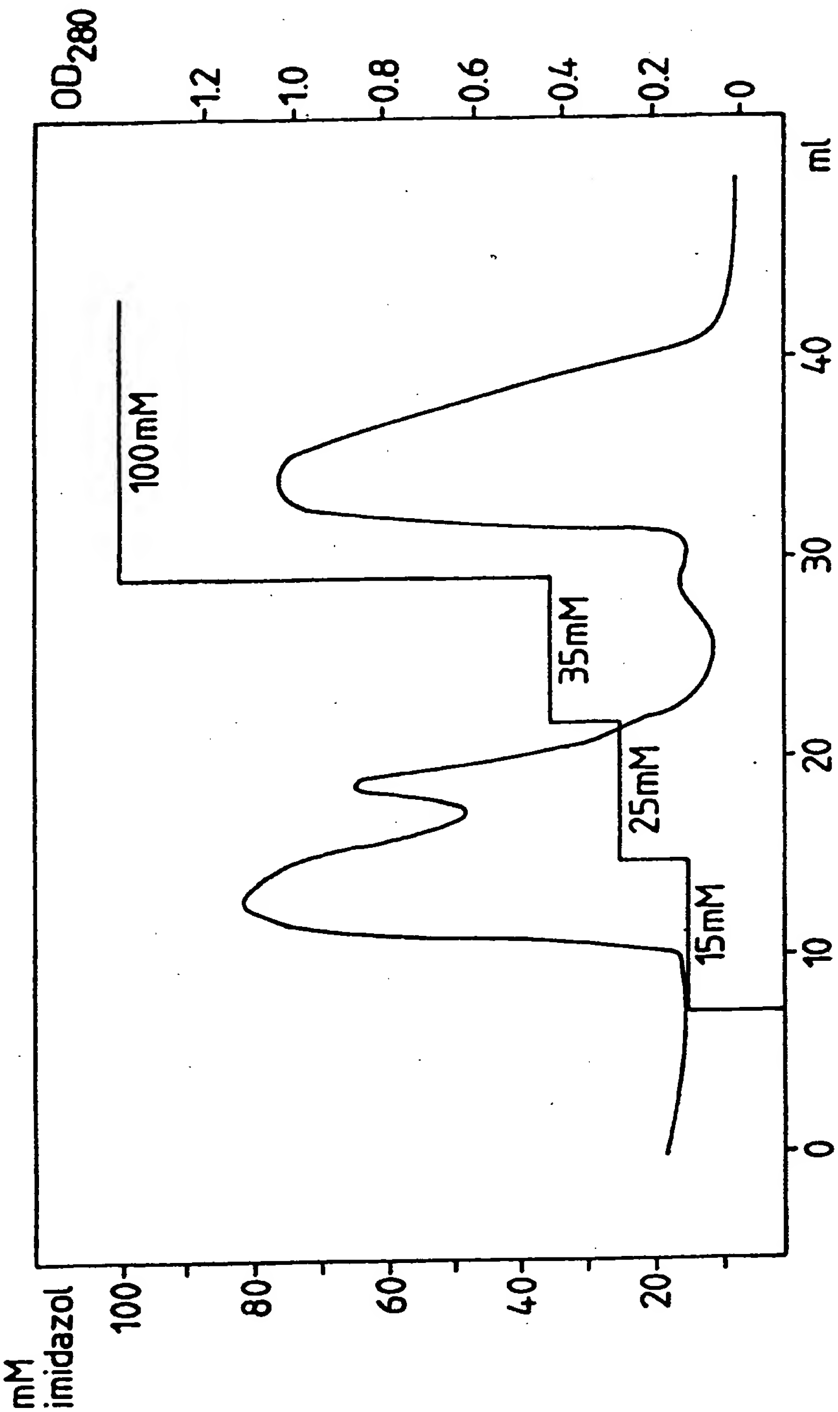
50
60
mM
imidazol
100
80
60
40
20

FIG. 16

FIG.17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25mM
15mM
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100
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60
40
20

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 90/01593

I. CLASSIFICATION F SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC^5: C 07 K 13/00, A 61 K 39/04, C 12 N 15/31, G 01 N 33/569
C 12 Q 1/68

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC^5	C 07 K, C 12 N, A 61 K, G 01 N, C 12 Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Chemical Abstracts, volume 99, 12 September 1983, (Columbus, Ohio, US), H. Tasaka et al.: "Purification and antigenic specificity of alpha protein (Yoneda and Fukui) from mycobacterium tuberculosis and mycobacterium intracellulare", see page 413, abstract 86251m & Hiroshima J. Med. Sci. 1983, 32(1), 1-8 (Eng). cited in the application ---	1-9,40,41
X	Journal of Clinical Microbiology, volume 25, no. 7, July 1987, M.L. Cohen et al.: " Expression of proteins of mycobacterium tuberculosis in escherichia coli and potential of recombinant genes and proteins for development of diagnostic reagents", page 1176 see the whole document cited in the application ./.	10-22,25-33, 35-39,43,44

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
20th December 1990	23 JAN 1991
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Mm N. KUIPER

International Application No PCT/EP 90/01593

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FR M THE SECOND SHEET)

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	BE, A, 905582 (INSTITUT PASTEUR) 9 April 1987 see pages 10,11; claims ---	35-39,45
X	Journal of Bacteriology, volume 170, no. 9, September 1988, Am. Soc. for Microbiology, K. Matsuo et al.: "Cloning and expression of the mycobacterium bovis BCG gene for extracellular α antigen pages 3847-3854 see the whole document	10-22,40,41, 43
Y		23,24,32, 34,42

Y	EP, A, 0288306 (McFADDEN) 26 October 1988 see page 7, column 12, lines 2-18 ---	23,24,32,34 42
A	Int. Archs Allergy appl. Immun, volume 81, 1986, S. Karger AG (Basel, CH), H.G. Wiker et al.: " MPB59, a widely cross-reacting protein of mycobacterium bovis BCG", page 307 ---	
A	Microbial Pathogenesis, volume 2, 1987, Academic Press Inc. (London GB) Ltd., J. De Bruyn et al.: " Purification characterization and identification of a 32 kDa protein antigen of mycobacterium bovis BCG", pages 351-366 ---	
P,X	Infection and Immunity, volume 57, no. 10, October 1989, American Society for Microbiology Pub.", M. Borremans et al.: "Cloning, sequence determination, and expression of a 32-kilodalton-protein gene of mycobacterium tuberculosis", pages 3123-3130 see the whole document ---------	1-45

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. EP 9001593 SA 40401

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 16/01/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
BE-A- 905582	09-04-87	None		
EP-A- 0288306	26-10-88	AU-A-	1628688	02-12-88
		EP-A-	0356450	07-03-90
		WO-A-	8808456	03-11-88

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Summary

Document	Pages	Printed	Missed	Copies
WO009104272	229	229	0	1
Total (1)	229	229	0	-

Figure 9a

```
            PROBE REGION A
1     ATG  CAGCTTGTTGACAGGGTTCGTGGCGCCGTCACGGGTATGTCGCGTCGACTC
      |||  ||||||||||||||||||||||||||||||||||||||||||||||||||
1     ATG  CAGCTTGTTGACAGGGTTCGTGGCGCCGTCACGGGTATGTCGCGTCGACTC
      |||         ||   | |        | | | | |   ||| | | || | || |
1     ATG  ACAGACGTGAGCCGAAAGATTCGAG CTT   GGGGACGCCG ATTGA TG

55    GTGGTCGGGGCCGTCGGCGCGGCCCTAGTGTCGGGTCTGGTCGGCGCCGTCGGTG
      |||||||||||||| |||||  ||||||||||||||||||||||||||||||||
55    GTGGTCGGGGCCGTC GCGCG   CCTAGTGTCGGGTCTGGTCGGCGCCGTCGGTG
       | | |  ||| |   ||       |  |  |||| ||||| ||     | ||| |
49    ATCGGCACGGCAGCG GCTGT   AGTCCTTCCGGGCCTGGTGGGGCTTGCCGGCG

                                                     P1
110   GCA CGGCGACCGCGGGGGCATTTTCCCGGCCGGGCTTGCCGGTG GAGTACCTG
      ||| |||||||||||||||||||||||||||||||||||||||||| |||||||||
107   GCA CGGCGACCGCGGGGGCATTTTCCCGGCCGGGCTTGCCGGTG GAGTACCTG
      |    |||| |||||||| || || |||||||||||   |||||||  |||||||||
101   GAG CGGCAACCGCGGGCGCGTTCTCCCGGCCGGGGCTGCCGGTC GAGTACCTG

163   CAGGTGCCGTCGCCGTCGATGGGCCG TGACATCAAGGTCCAATTCCAAAGTGGT
      |||||||||||||||||||||||||| |||||||||||||||||||||||||||
160   CAGGTGCCGTCGCCGTCGATGGGCCG TGACATCAAGGTCCAATTCCAAAGTGGT
      ||||||||||||||||||||||||||  |||||||||| || ||||| || |||
154   CAGGTGCCGTCGCCGTCGATGGGCCG CGACATCAAGGTTCAGTTCCAGAGCGGT

                 PROBE REGION B
217   GGTGCCAAC TCGCCCGCCCTGTACCTG CTCGACGGCCTGCGCGCGCAGGACGA
      ||||||||| |||||||||||||||||| ||||||||||||||||||||||||||
214   GGTGCCAAC TCGCCCGCCCTGTACCTG CTCGACGGCCTGCGCGCGCAGGACGA
      ||  |||| || || ||  | || ||| |||||||||||||||| || |||||
208   GGGAACAAC TCACCTGCGGTTTATCTG CTCGACGGCCTGCGCGCCCAAGACGA
```

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